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NASA Technical Memorandum 4160

1988–89 NASA Space/Gravitational Biology Accomplishments

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Washington, D.C.

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1990

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PREFACE

An individual technical summary of each research task within the NASA Space/Gravitational Biology Program is presented in this publication. Each summary, prepared by the principal investigator, consists of a description of the research, a listing of the project's accomplishments, an explanation of the significance of the accomplishments, and a list of the publications resulting from the past year's research. Since spaceflight experiments, submitted in response to the Space Biology Dear Colleague letter, have become an integral part of the Program, reports on the activites of this related research are integrated in the report. Accomplishments of the scientists in the NASA Space Biology Research Associates Program, which provides opportunities for postdoctoral scientists to conduct research in the fields of gravitational and space biology, and the NASA Graduate Student Researchers Program, which supports promising students pursuing advanced degrees in science and engineering, are also included. The participants in these programs have been outstanding and merit independent recognition.

This publication has two objectives: first, to provide the scientific community and NASA with an annual summary of the accomplishments of the research pursued under the auspices of the Space/Gravitational Biology Program, and second, to stimulate an exchange of information and ideas among scientists working in the fields of gravitational and space biology.

Thanks are due to the Program participants and postdoctoral and graduate student scientists whose research and cooperative response to our requests for information made this report possible. Editorial support provided by Janet V. Powers, Katherine J. Dickson, and F. Ronald Dutcher is gratefully acknowledged and appreciated, as well as the technical assistance provided by April Commodore Roy.

Additional information about this report of the Space/Gravitational Biology Program can be obtained by writing to me at the following address:

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THE NASA SPACE/GRAVITATIONAL BIOLOGY PROGRAM

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Introduction

One of the major features of the physical environment of the surface of Earth is the constant presence of the force of gravity. The phenomenon of weightlessness encountered on spacecraft provides a unique biological research opportunity to study the importance of gravity to life on Earth. Access to space provides an opportunity to manipulate gravity from its norm of one down to almost zero, effectively providing the full spectrum of gravitational research capability for the first time. This capability, combined with the stability and pervasiveness of gravity on Earth, its obvious impact on biological evolution, and its continuing effect on the morphology, physiology, and behavior of living organisms, has led the Space/Gravitational Biology Program to concentrate its efforts and resources on investigating the biological significance of gravity and advancing knowledge in the biological sciences through the use of the microgravity environment of spaceflight.

Program Goals

The goals of the Space/Gravitational Biology Program are to: use the unique characteristics of the space environment, particularly microgravity, as a tool to advance knowledge in the biological sciences; understand the role of gravity in the biological processes of both plants and animals; and understand how plants and animals are affected by and adapt to the spaceflight environment, thereby enhancing our capability to use and explore space.

Program Scope

Research in the Program is divided into four broad areas:

1. **Gravity Perception/Sensing.** Plants and animals have developed gravity-sensing systems that facilitate orientation and locomotion within Earth's environment. The weightless environment of space provides a unique research opportunity to understand how gravity-sensing systems of different organisms have developed, and how they process and transmit information. Specific objectives are:
    a. To identify gravity-sensing organs and mechanisms, and to define how they function on Earth and adapt to weightlessness.
    b. To understand how gravitational information is transduced, processed, transmitted, and integrated into a response.
    c. To understand the role of gravity in the development and evolution of plant and animal gravity sensing systems.

2. **Developmental Biology.** Research in this area examines the influence of gravity and weightlessness on genetic integrity, reproduction, growth, development, life span, senescence, and subsequent generations of plants and animals. Specific objectives are:
    a. To determine if organisms and multiple generations of organisms can develop normally in microgravity.

b. To identify gravity-sensitive developmental stages, systems, and mechanisms in both plants and animals.
c. To understand the effects of gravity and weightlessness on gravity-sensitive developmental stages, systems, and mechanisms.

3. **Biological Adaptation**. All biological species on Earth have evolved under the influence of a gravity level of 1 g. In response to this force, organisms have developed structures to withstand gravity loads, as well as regulatory systems to function optimally. The objective is to understand how gravity affects and controls the physiology, morphology, and behavior of organisms; how gravity and other environmental stimuli and stresses interact in this control; and the biological mechanisms by which living systems can respond and adapt to altered gravity, particularly that of the space environment. It includes the use or removal of gravity's physiological effects to explore biological problems. Specific objectives are:
    a. To understand the influence of gravity on the composition, regulation, and function of biological support structures.
    b. To determine the role of gravity in regulating metabolism, metabolic rate and products, fluid dynamics, and biorhythms.
    c. To understand basic mechanisms of mineral and hormonal homeostasis and the role of calcium as a mediator of gravitational effects.
    d. To identify the effects on organisms of the interaction of environmental factors (e.g., temperature and light) with gravity, and determine the mechanisms involved.

4. **Cell Biology**. Cells that are building blocks of systems (e.g., plant root caps), individually functioning units of certain tissues (e.g., blood cells), and unicellular organisms (e.g., paramecia) have been shown to be sensitive to gravity. Research focuses on how gravitational loading influences cell functions and the molecular mechanisms regulating them. The objective is to determine at what level gravity affects cells and where and how they are affected. Specific objectives are:
    a. To investigate the role of gravity in maintaining normal cellular and molecular function.
    b. To determine how and where gravity affects cells.
    c. To distinguish direct from indirect, extracellular, or systemic, gravitational effects on cells.
    d. To discriminate between the influences of cosmic rays, microgravity, and other environmental factors.
    e. To assess the permanence of effects on cells exposed to microgravity.

Focus of Program

The program focuses on research that promises to answer basic scientific questions that can contribute to the resolution of biological problems of fundamental importance on Earth and/or to space exploration.

Understanding how plants develop, metabolize and grow in space is essential for a space based Controlled Ecological Life Support System (CELSS). Biomineralization and the mechanisms controlling the structural integrity of bone and bone turnover are important to osteoporosis on Earth and the calcium loss and bone changes of spaceflight. The reconstruction and modeling of the functional organization of mammalian gravity sensors

are expected to lead to increased understanding of how information is processed by biological systems. In a sense this is all basic research with an eye on application.

Research Opportunities

While the research supported and encompassed by the Space/Gravitational Biology Program is primarily ground-based, spaceflight experiments are an essential component of the program. Spaceflight provides the validation for experimental hypotheses developed in ground-based research, while gravitational experiments on Earth hone the questions, provide the necessary baseline data, and develop spaceflight experimental protocol.

The experimental approach of the ground-based studies is to manipulate gravity on Earth and develop weightless simulation models to: (a) develop and test gravitational hypotheses, (b) identify gravity-sensitive biological systems and interacting environmental response mechanisms, (c) analyze biological systems and mechanisms known to be gravity-sensitive, (d) analyze flight experiment data and iteratively expand ground research capability, and (e) plan and design future space experiments. Research is needed, but currently not funded, to understand how the uncontrollable biodynamic factors of the spacecraft and behavior of components of the environment in weightlessness affect the results of flight experiments.

The Space Shuttle is currently the only U.S. developed spacecraft capable of carrying biological experiments. This limited opportunity to conduct biological experiments on spacecraft has stimulated the examination of alternative means to conduct space research. Biosatellites offer such an alternative. In fact, they offer a valuable supplemental capability. While limited in the extent of experimental manipulation that can be achieved, they have the significant advantage of extended stay in space at a low microgravity level of 10^{-5} g. Currently a retrievable reusable biosatellite called LifeSat is under study with the support of international space agencies.

The research of the Space/Gravitational Biology Program is dependent upon several dynamic factors: the requirements of NASA, the characteristics of flight experiment opportunities, the sensitivity of specific biological systems to gravity, the scientific value of the research, the state of knowledge and technology in the specific scientific areas, the interest of scientists in studying the biological questions, and the availability of funds to support the research. Of these factors, scientific interest in this field of biological science is paramount; for without outspoken support from the scientific community, opportunities for space/gravitational research will remain static.

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ACCOMPLISHMENT HIGHLIGHTS

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SPACE/GRAVITATIONAL BIOLOGY
ACCOMPLISHMENT HIGHLIGHTS

PLANT

Gravitropism: Sensing

- Nature of the receptor
    - A tobacco mutant containing reduced starch is less responsive to gravity, which is additional evidence for amyloplasts as the gravity sensor in plants. (Sack)
    - In moss filaments responding to gravity, the cell outline at the filament tip changes within minutes. An initial "wrong-way" curvature, which can occur in higher plants, often occurs in moss filaments as well. (Sack)
    - Amyloplast sedimentation occurs in moss filaments before upward filament curvature begins. The same cell perceives and responds to gravity in this system. (Sack)
    - Reorientation of the moss filaments results in an increase in microtubule density close to the part of the cell on the lower half where curvature develops. This is the first report of an effect of gravity on cytoskeletal organization in plants. The microtubules are closely associated with amyloplasts. (Sack)
    - An analysis of the energetics of gravity receptor activation in higher plant roots suggests that the observed behavior of amyloplasts is in line with the predicted value. (Björkman)
- Membrane channels
    - Patch clamp analysis has revealed several channels in isolated membranes of the upright sporangiophore of the fungus *Phycomyces*, an organism that exhibits a gravitropic response. (Edwards)
    - Gadolinium, which inhibits gravitropism of higher plant roots and shoots and which inhibits stretch-activated membrane channels in amphibian oocytes, inhibits gravitropism in *Phycomyces* as well. (Edwards)

Gravitropism: Transduction Mechanism

- Role of calcium
    - Preliminary data for direct measurements with a calcium microelectrode of calcium activity in gravistimulated corn root tips suggest a small decline in calcium activity on the upper side of the root tip. (Björkman)
    - The free level of calcium in the cytoplasm of protoplasts derived from the root elongation zone (where gravitropic bending occurs) is much higher than in protoplasts derived from the root cap (where gravity is sensed). (Evans)

- Roots that grow horizontally and that do not respond to gravity (diagravitropic) are less sensitive to calcium availability and have a lower calcium requirement than roots that respond normally to gravity. (Leopold)

- Rapid *in vivo* calcium- and calmodulin-dependent changes in protein phosphorylation have been observed in corn roots, primarily in the root tips. (Poovaiah)

- A plant calmodulin cDNA was recently cloned and sequenced. Changes in calmodulin gene expression in root tips have been observed. (Poovaiah)

- A calmodulin-dependent protein kinase has been isolated in corn. (Poovaiah)

- Messenger RNAs for kinases (proteins hypothesized to be involved in processing of the gravity signal by phosphorylating proteins) were shown to be present in the root cap, and in adjacent root tissues. (Feldman)

- Role of hormones

  - Auxin-binding membrane proteins have been photoaffinity labelled and identified in zucchini hypocotyls using the auxin analogue azido-IAA, suggesting that these polypeptides are part of the auxin receptor system. Furthermore, shoots of the tomato mutant *diageotropica*, which is auxin non-responsive and is also altered in its gravity response, lack these polypeptides. (Lomax; Rayle)

  - In corn seedlings, the point at which gravity exerts control is in the movement of IAA from stele (inner ring of conductive tissue) to cortex. (Bandurski)

  - Communication across the stele:cortex interface appears to occur through the symplast (the living component of plant tissues), rather than through the apoplast (the nonliving component). (Desrosiers)

  - The movement of solutes from stele to cortex apparently occurs through small intercellular connections called plasmodesmata. (Bandurski)

  - Electrical impedance measurements suggest there are more open stele-to-cortex transport channels (presumably hormone transport channels) in the active growing region of corn shoots, compared with the rest of the shoot. (Desrosiers)

  - Gravitropic curvature will occur in front of but not behind a section of the root elongation zone where a narrow ring of cortical tissue has been removed, indicating that the gravitropic signal from root cap to elongation zone passes through the root cortex and/or epidermis. (Evans)

  - The ability of ethylene to cause exaggerated gravitropic curvature in corn roots appears to be the result of retardation of adaptation to the gravistimulus. This is the first evidence for a hormonal effect on the timing of adaptation to gravistimulation. (Evans)

- Experiments in which mucilage was removed from and applied to corn roots suggest that gravitropic effectors can move apoplastically, that is, in the nonliving component of plants. (Moore)

- The apoplast may be an important part of the gravitropic effector pathway between the cap and the root. (Moore)

- Excision of the node from cereal grass pulvini eliminates the gravitropic response. Since the pulvinus is known to be the site of gravity perception in cereal grasses, this constitutes a system for separating gravity perception from the gravity response. (Kaufman)

- Polar transport of labeled IAA in the cereal grass pulvinus declines upon gravistimulation, indicating a linkage between polar auxin transport and pulvinus orientation or graviperception. Lateral transport of IAA occurs with gravistimulation. (Kaufman)

- Attempts to find potential sources of error in experiments indicating that the role of auxin in gravitropism may be due to changed sensitivity to auxin have failed to find any such sources of error. (Salisbury)

- Oxidation of IAA to oxindole-3-acetic acid (a step whereby the growth-controlling IAA gradient may be regulated by inactivation of IAA) has been shown to be mediated by a novel enzyme. (Reinecke)

- The enzyme that oxidizes IAA and its cofactor is being characterized. The enzyme occurs not only during germination but also in green vegetative seedlings. (Reinecke)

- Relationship to light effects

    - Abscisic acid applied to corn roots can entirely substitute for the red light stimulation required for some roots before gravitropism can occur. Red light also causes an increase in abscisic acid in these roots. (Leopold)

    - Since abscisic acid seems to increase the concentration of calcium in plant cells, the red light effect may act by altering the calcium availability in the cytoplasm of gravity sensing or transducing cells. (Leopold)

    - Light affects the levels of many messenger RNAs and proteins in roots that require light for gravitropism to occur. There does not seem to be a single specific gravity transduction protein. (Feldman)

    - Light effects on calmodulin messenger RNA are not confined to the root cap, but rather occur throughout the root tip, which suggests that it is unlikely that light influences root gravitropism via alterations in calmodulin. (Feldman)

    - The effects of light may be more general and may extend beyond simply influencing events within the root cap. (Feldman)

Gravitropism: Growth Response

- Calcium crosslinks do not serve as significant stiffening agents in cell walls of plant shoots. Breakage of these links with protons is not the cause of selective wall loosening during gravitropic stem bending. (Cleland)

- Redistribution of total or bulk calcium between upper and lower sides of stems does not occur during gravitropism. (Cleland; Cosgrove)

- Calcium applied at physiologically meaningful levels does not inhibit cell expansion or rigidify the cell wall. (Cosgrove)

- Very high levels of external calcium do not interfere with the normal gravitropic response. (Cosgrove)

- The preceding three results are evidence against the idea that changes in extracellular calcium play an important role in the transduction/response mechanisms of gravitropism. (Cosgrove)

- Gravistimulation results in increases in invertase activity and the level of beta-D-glucan (a cell wall polysaccharide) in the lower halves of cereal grass pulvini. Beta-D-glucan may serve as a site of action of hormones such as IAA as they control differential cell wall loosening and synthesis in this system. (Kaufman)

- Changes in a 98 kDa wall peroxidase precede and correspond with changes in growth of both coleoptiles and mesocotyls exposed to red light, which suggests that the peroxidase could participate in transduction of the light signal into growth changes. (Roux)

- A 60 kDa cellulase has been purified from the cell wall of corn seedlings. (Roux)

- Application of hydrogen peroxide, which may play a role in gravitropic plant bending by serving as a substrate for peroxidase reactions involved in wall cross-linking, induces differential growth in corn seedlings. (Slocum)

- A sensitive method for measuring polyamines in plant tissues has been developed. Polyamines may serve as a starting point in a chain of reactions that may lead to peroxidase-mediated wall cross-linking. (Slocum)

Physiology/Metabolism

- Examination of onion and spinach seedlings grown in space confirm the result obtained earlier with corn seedlings that microgravity significantly affects the structure and function of plant cells. (Moore)

- Clinostating does not appear to mimic the effects of microgravity in terms of plant cell ultrastructure. (Moore)

- Among a variety of radiolabelled lignin precursors, the most efficient incorporation into lignified portions of leaf, stem, and root tissue was obtained with phenylalanine. (Lewis)

Environmental Factors

- Single brief episodes of mechanical vibration or rubbing of pea seedlings causes an abrupt drop in growth rate within minutes. This is a sensitive and rapid response. (Mitchell)

- After 24 hours, there is often a net growth stimulation following vibration but a net growth inhibition following rubbing. (Mitchell)

- Rubbing inhibits auxin transport in pea stems to the same extent that it inhibits growth of stems over a 24-hour period. (Mitchell)

- Mechanical stress increases plant cell wall extensibility, particularly the elastic component of wall extensibility. (Mitchell)

Circadian Rhythms

- The effects of temperature on the circadian rhythm of conidiation in *Neurospora* have been characterized. (Ferraro)

- The optimal temperature range for the *Neurospora* circadian rhythm experiment scheduled to fly on STS-32 in November 1989 has been determined. (Ferraro)

Plant Cell & Developmental Biology

- Molecular characterization of a yeast gene whose protein is involved in regulation of chromosome segregation during cell division has been accomplished. (Bruschi)

- Behavior of chromosomes carrying a mutation in the yeast chromosome segregation gene has been characterized, including loss of a chromosome, addition of a second copy of the missing chromosome, and the occurrence of complex chromosomal aberrations. (Bruschi)

- A system for producing somatic embryos directly from seed tissue of carrot without any exposure to exogenously added growth regulators has been developed; it should serve as a model system for examining the effect of environmental factors on early plant development. (Krikorian)

ANIMAL

Gravity Receptors and Neurophysiology

- Three-dimensional reconstructions of macular receptive fields and portions of the neural network have been generated. The connectivities of more than 200 cells have been mapped, and the results show that both type I and type II hair cells comprise receptive fields, and that there are three basic configurations of fields and three kinds of nerve patterns. (Ross)

- Macular receptive fields are morphologically organized for weighted, parallel distributed processing of information. This processing is subject to intrinsic modulation by calyceal and nerve collaterals. (Ross)

- Wiring of macular receptive fields is probably more the result of developmentally introduced randomness rather than strict genetic control. (Ross)

- The hyper-g challenge to the derhopaliaized *Aurelia* (jellyfish) ephyrae during parabolic flight demonstrated that, without graviceptors, the organism cannot be stimulated to pulsing/swimming activity. (Spangenberg)

- For the first time, the growth and maturation of ephyra graviceptors into medusa-type graviceptors has been achieved in a simple liquid nutrient medium. (Spangenberg)

- The time course of production of statoconia in molluscs has been determined. Multiple statoconia are produced in the supporting cells and production begins when the statocyte grows to the size at which the cilia can no longer support the statolith. (Wiederhold)

- Antibodies have been generated against the specific proteins from rat and frog otoconia. (Pote)

- Physiologic responses of otolith afferents in adult rats reveal predominantly "tonic" responses and few "phasic-tonic" responses. In addition, otolith afferents respond in an essentially linear manner to head displacement and independently of stimulus amplitude. (Blanks)

- Horizontal canal afferent (HCA) fiber responses to mechanical and rotational stimulation can be directly equated. The response dynamics produced by the two methods of stimulation are practically identical. (Dickman)

- Using intracellular dye to trace functionally characterized vestibular afferent axons in bullfrogs to their peripheral origins, anterior ventrical canal axons have been definitively separated from utricular axons and show that the traditional functional criteria are inadequate for that purpose. (Lewis)

- Rotation sensors are traditionally thought to be semicircular canals, but observations of gravity-mediated rotational velocity in bullfrogs imply that the faithful rotational velocity sense arises from the (phasic) hair cells at the very center of the utricular striola. Furthermore, results suggest that serious deficits in rotational motion sensitivity could occur in microgravity environments. (Lewis)

- Anatomical track tracing experiments in the larval frog reveal that many cells throughout the brain project to the oculomotor neurons, most notably second order

vestibular neurons and cells in the cerebellar peduncles. Preliminary experiments indicate that the activity of the oculomotor neurons can be modulated by lateral head tilt. In addition, electrical stimulation of either VIIIth nerve can activate these cells. (Cochran)

- Responses to pulsed linear acceleration in mammals and birds appear to depend on neurons that are more sensitive to the rate of change in acceleration than acceleration itself. (Jones)

Development

- A new class of amphibian eggs that display a preprogrammed bilateral symmetry has been indentified. Their polarity is not determined by the sperm entry point but by a built-in gravitational bias (tilt of the egg's animal/vegetal axis with respect to the gravity vector). (Malacinski)

- Cytoplasmic mobility (CM) can be correlated with the egg's gravitational response. There is a direct correlation between CM and inverted egg survival, primordial germ cell members, and failure to gastrulate properly. (Malacinski)

- It has been found that amphibian embryological cells destined to become neural are biased in this direction much earlier in their development than previously believed. A small group of cells, which eventually form the initiation site for gastrulation, have the ability to pattern subsequent neural development. (Phillips)

- Development in a slow clinostat (1-10 rpm) results in a dramatic reduction of the appearance of nerve-induced acetylcholine receptor accumulation at the point of contact between nerve and muscle cells in culture. (Gruener)

- Mice reared from birth on a centrifuge at 2.3 and 2.6 g are less sensitive to a decreased gravitational field, i.e., return to 1 g Earth gravity, than are mice placed on a centrifuge at 4 weeks of age. (Duke)

Bone and Muscle

- Analysis of Cosmos 1887 rat tissues showing major defects in tibial periosteal vascularity suggests that microvascularity at the surface of the bone shaft is altered by spaceflight and might be associated with, or perhaps even initiate, the changes in bone maturation. (Morey-Holton)

- Structural damage to the endothelial cells and a reduction in some enzyme activities was observed in bones of rats following the Cosmos 1887 spaceflight. In addition, there was an asymmetrical distribution of damaged vessels in the outer third of the long bones, while no damage was noted in the marrow or vessels at the growing ends of the long bones. (Doty)

- Recovery of preosteoblast formation occurred in the periodontal ligament of rat bones following flight onboard Cosmos 1887 despite a highly significant degree of physiological stress. (Roberts)

- Bone formation in a culture system exhibits biochemical and ultrastructural characteristics similar, if not identical, to those of embryonic bone *in vivo*. (Landis)

- Extracellular matrix formation is dependent on post-translational events affecting collagen accumulation and not collagen synthesis per se. Collagen accumulation is not dependent on the rate of synthesis but may also be dependent on the efficiency of collagen fibril formation. (Landis)

- Bone cells grown in culture on a three dimensional lattice made of beads appear to differentiate more rapidly than those grown on a standard flat surface. (Doty)

- Under certain culture conditions, rat osteoblast-like cells grown on collagen-coated beads become phagocytic, rather than producing bone matrix, and actually engulf part of the collagen bead. Thus, these cells are capable of both degradation and synthesis. (Morey-Holton)

- Roller bottle cultures cause osteoblastic cells to produce greater amounts of proteins associate with bone formation and lesser amounts of proteins associated with bone breakdown. This indicates osteoblast responsiveness to motion and/or gravity. (Partridge)

- Glucocorticoids decrease: prostaglandin synthesis, protein synthesis and DNA synthesis in growing osteoblasts; the number of proliferating bone osteoblasts; and the synthesis of procollagen and its post processing to collagen. These results suggest that glucocorticoids may inhibit bone formation, both by interfereing with the growth of the osteocyte and by inhibiting synthesis of the collagen matrix. (Hughes-Fulford)

- The major rate-limiting step in preosteoblast formation is probably mediated by prostaglandins and the block occurs after induction of the A' ---> C shift. (Roberts)

- The application of 15 Hz loads to produce a strain of 500 microstrain to an avian ulna preparation prevents normal bone loss and induces substantial new bone formation. (McLeod)

- Vitamin D affects the differentiation of both early and intermediate bone marrow osteoclast precursor cells. (Perkins)

- Treatment with parathyroid hormone appears to change the phenotype of the osteoblast from a matrix-synthesizing cell to one actively involved in the resorption process. The hormone induces increased collagenase mRNA, suggesting an effect of the hormone at the gene level. (Partridge)

- Hypodynamia structurally changes sub-cellular components which control functional capacity of skeletal muscle in rats. The presence of Targetoid type fibers in the reloaded soleus muscle of rats recovering from hypokinesia indicates a predisposition of the atrophied postural muscle to reuse injury. (Kasper)

- The β-adrenergic binding capacity of the soleus increases markedly following three days of unloading. (Tischler)

- Blocking the glucocorticoid receptor in unloaded soleus muscles of rats does not change the effect of unloading on muscle protein content or protein synthesis, but does lead to less atrophy in control muscle by greater growth due to slower protein degradation. This indicates that glucocorticoids are not the cause of atrophy in unloaded soleus muscle, though this hormone may exacerbate the atrophy. (Tischler)

Regulatory Biology

- Core temperature has been found to be regulated in rats exposed to hypergravic fields. Though the thermoregulatory system is impaired in hypergravic fields, and transiently falls several degrees centigrade, the rat then activates thermoregulatory mechanisms to control core temperature. (Horowitz)

- In a normal 12:12 light/dark cycle, rats exhibit reductions in the amplitudes of heart rate and activity rhythms following exposure to 2 g. They also exhibit recovery of body temperature rhythm amplitude earlier than rats in a constant light environment. (Fuller)

- Lesions of the ventromedial nuclei of the hypothalamus reduce the increases in plasma renin activity produced by immobilization, head-up tilt, and a low sodium diet, but do not reduce levels of circulating angiotensinogen. Renin responses to immobilization and head-up tilt are blocked by the β-adrenergic blocking drug propranolol. These results indicate that the final common pathway from the spinal cord to the renin-secreting cells is sympathetic. (Ganong)

- Systemically administered vasopressin inhibits renin secretion, possibly by a direct effect on the renin secreting cells in the kidney and possibly by a baroreceptor reflex. (Ganong)

- In addition to regulating angiotensinogen secretion, the paraventricular nuclei of the hypothalamus are involved in the regulation of the secretion of renin from the kidneys in some situations. (Ganong)

- Unloading with or without head-down tilt causes specific changes in immune function in coordination with decreased circulating $1,25(OH)_2$ D. This modeling has selective immunological effects rather than an overall immunosuppression. Spleen cell production of Interleukin 1 (IL-1) is reduced with unloading in rats fed on a vitamin D deficient diet. (Berry)

Cell Biology

- The release of bioactive growth hormone (GH) from pituitary cells of rats after flight onboard Cosmos 1887 was compromised, thus confirming results of Spacelab 3. (Hymer)

- It has been found that not all GH secreting cells produce a hormone with equal biological potency and that these cells are asymmetrically distributed within the pituitary gland. (Hymer)

- It has been shown that fibronectin modulates cell growth by: 1) binding to cell surface integrin receptors, 2) promoting cell spreading, and 3) facilitating nuclear extension within cells attached to rigid, planar substrata. (Ingber)

- Results of studies with attached cells clearly confirm that the growth and differentiation-modulating effects of extracellular matrix (ECM) components depend upon their ability to provide a physical anchoring substratum that can resist cell-generated tensile forces and support cell extension. (Ingber)

- Interactions between ECM molecules and their cell surface receptors may serve to alter cell function by transducing externally applied mechanical loads into changes of cytoskeletal structure. (Ingber)

PLANT PROJECTS

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LOCALIZATION AND IDENTIFICATION OF THE GRAVITY SENSING MECHANISM OF PLANTS

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Description of Research

A gravitational stimulus as, for example, moving a plant shoot from a vertical to a horizontal orientation relative to the gravitational vector, induces an asymmetric distribution of indole-3-acetic acid (IAA) and calcium. Both chemical asymmetries occur within minutes after the gravity stimulus. They are preceded by a membrane depolarization which occurs within seconds following the stimulus. From these facts we adduced the following postulates as parts of a working theory for the transduction of the gravitational stimulus: (1) the gravity stimulus causes membrane depolarization; (2) the membrane depolarization opens and/or closes gated channels connecting the plant's vascular stele with the surrounding cortical tissues; (3) IAA and/or its conjugates, which are transported from seed to shoot through the stele, move out of the stele and selectively into the cortex of the lower side of the horizontal shoot; (4) the resultant hormone asymmetry induces a growth asymmetry resulting in the tropic growth response; (5) vertical growth is attained after hormone metabolism equalizes the amount of hormone on the two sides of the tissues.

This laboratory has concentrated on the question of how the chemical asymmetry is attained and subsequently corrected. We believe that it will be possible to attain an understanding of the gravity response at the molecular level by concentrating on the mechanism by which the chemical asymmetry is attained.

Accomplishments

From the above discussion it appears there are two phases to the control of IAA levels: (1) the release of IAA from the stele into the surrounding cortical tissues; and (2) the metabolism of IAA after it enters the target cortical and epidermal cells.

(1) ***IAA release:***

(a) Free radioactive IAA in the endosperm does not appreciably label the free IAA pool of the coleoptile. This finding plus our earlier studies on rate of transport of IAA and IAA-*myo*-inositol from kernel to shoot indicates that IAA-*myo*-inositol leaves the endosperm, travels through the vascular stele, and is then hydrolyzed either in the stele or in the cortex to appear as free IAA in the cortex. ***The point at which gravity exerts control is in the movement of IAA from stele to cortex.***

(b) We have demonstrated that ***there are no apoplastic connections between the stele and cortex of the mesocotyl.*** This is evidenced by the failure of light green (a dye with three sulfonic acid groups and one acidic hydroxyl group) to move from stele to cortex (Epel and Bandurski, *Plant Physiology* 83(Suppl.): 66; Epel and Bandurski, submitted).

(c) Figure 1 shows some of the plasmodesmata connecting the symplast of the stele with the symplast of the cortex. There is no apoplastic communication between stele and cortex and thus ***the movement of solutes from stele to cortex must occur through the plasmodesmata.*** The gated channels through which solutes move from stele to cortex would occur in the plasmodesmata as shown in this figure.

PLASMODESMATA CONNECTING STELE AND CORTEX

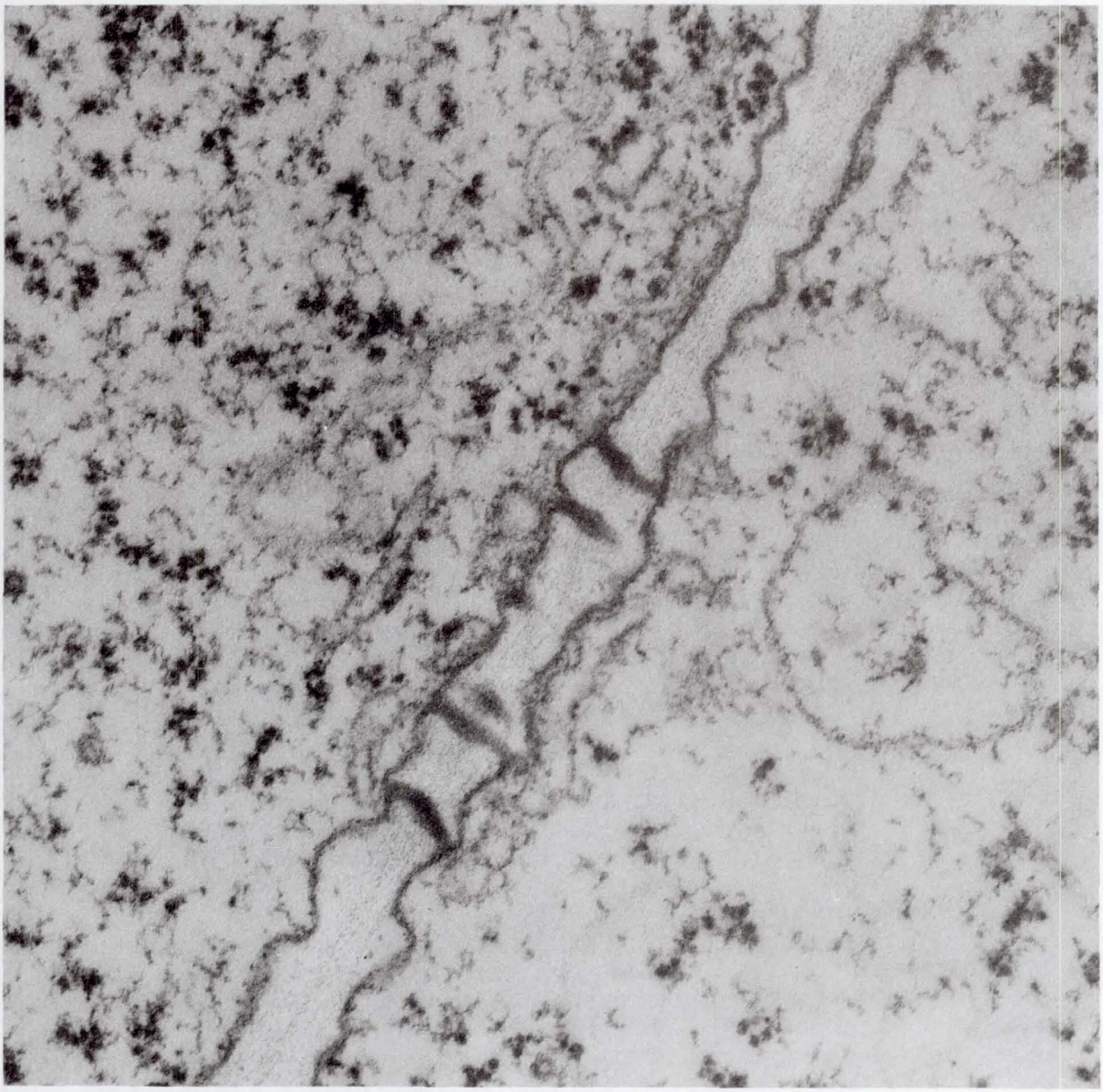

Figure 1. Longitudinal section of plasmodesmata between endodermal and pericycle cells of the mesocotyl (located between 4 to 14 mm below the coleoptile-mesocotyl node) of a *Zea mays* seedling. (Courtesy of Dr. Robert Warmbrodt.)

(2) ***Metabolism of IAA***

(a) Greatly simplifying our studies is the finding of Jensen that seedling corn plants *do not* incorporate deuterium into non-exchangeable positions of the indole ring when the plants are grown on 30% deuterium oxide. This is true for the first two weeks of growth and since our experiments utilize 5-day old plants, we are free of the complexities of *de novo* synthesis. ***The plants under study are utilizing their reserves of IAA esters.***

(b) Dr. Stanley Kowalczyk has succeeded in purifying to homogeneity the enzyme catalyzing the synthesis of indole-3-acetyl-1-0-ß-D-glucopyranoside (IAGlu) from IAA and uridinediphosphoglucose (UDPG). This is the first step in the series of reactions leading to the synthesis of the esters of IAA (Figure 2). Understanding the control of this reaction, in which a growth promoting active IAA is converted into an inactive conjugate, is vital to understanding how a plant stops bending once it has attained a vertical orientation.

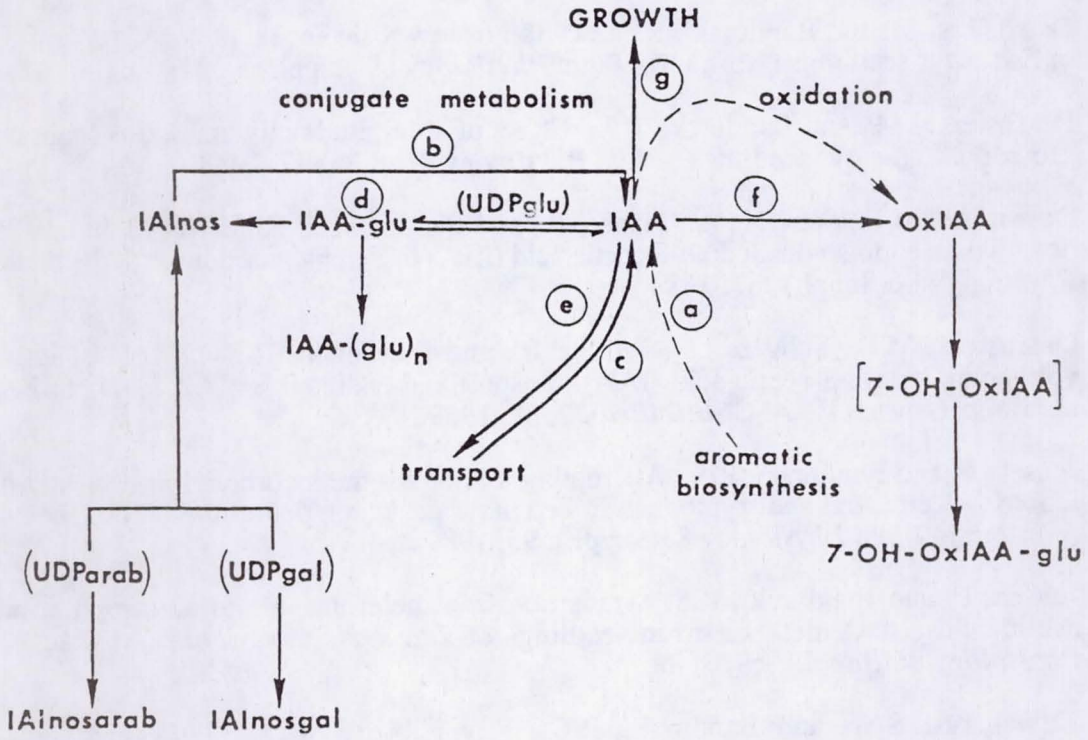

Figure 2. The "inputs to" and the "outputs from" the IAA pool determine the steady state level of IAA in a particular plant tissue (e.g., corn). IAA inputs include: a) *de novo* synthesis; b) conjugate hydrolysis; and c) transport. IAA outputs include: d) conjugate synthesis; f) catabolic oxidation; g) IAA "use" during the growth process; and e) transport.

Significance of the Accomplishments

If our theory is correct, then we have found the target of the gravity stimulus — that is, the gates within the plasmodesmata that control the movement of IAA from the vascular stele into the surrounding cortical tissues.

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DETERMINING THE ROLE OF GRAVITY IN PLANT PHYSIOLOGY AND DEVELOPMENT

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Description of Research

The long-range research goal is to improve our understanding of how gravity is important for plant development and physiological behavior. The unique advantage offered by experimental access to protracted microgravity is the ability to observe plant growth and functions and to perform experiments in protracted hypogravity. To accomplish these goals it is necessary to use not only an orbiting laboratory, but also a centrifuge of suitable design so that tests can be conducted throughout the g-force region from essentially zero to unit g and above. Both exploratory research investigations and critical tests of theory have been the subjects of our past and hopefully some future studies in true hypogravity. Our prediction that research on plants in hypogravity would be scientifically fruitful and would produce some surprise already has proven to be correct, which disposes us to be optimistic about future scientific experimentation with plants in space laboratories.

Experimental manipulation of the g-force magnitude is unambiguously achievable in space. Flight opportunities are infrequent. Therefore, utilization of hypergravity (centrifugation) and of simulated hypogravity (clinostat rotation), both conveniently attainable in earth-bound laboratories, have been exploited often as preliminary to a definitive flight experiment. Since our experimental materials (seedlings and small plants) usually are too large for appropriate use of fast rotating clinostats (ca. 0.8 Hz), we use slowly rotating clinostats (ca. 1.7 to 17 mHz). Much if not most of our work with clinostats requires rotation simultaneously on two orthogonal axes, one of which must be as nearly as possible horizontal. In some cases comparison of results from clinostat simulation experiments and from space experiments (preferably with the same apparatus) can be used to test the validity of the simulation method. Whenever possible we try to plan experiments in such a way that this kind of validation test can be achieved, if only as a secondary but none the less very important scientific objective.

Accomplishments

In preparation for participation in the first International Microgravity Laboratory (IML-1) mission, now scheduled for launch in December 1990, we have developed and tested the flight hardware, called the Gravitational Plant Physiology Facility (GPPF), which contains integrated equipment for two experiments, FOTRAN and GTHRES. The tests involved the following activities: (a) Developed a refined system for analysis of GTHRES and FOTRAN images in near realtime during flight. This system was designed and perfected with assistance with co-investigator Dr. Anders Johnsson of Trondheim, Norway; (b) Conducted a second series of investigator ground studies (IGS-2) at the NASA Ames Research Center where the flight hardware was shipped after fabrication and preliminary testing in our Philadephia laboratory. IGS-2 collected data from 16 GTHRES test sequences and 7 FOTRAN test sequences. The primary purpose of these tests was to collect ground based data from both experimental components of GPPF for later comparison with data to be collected in orbit at different hypogravity g levels. A secondary

objective was to obtain Earth operational information on the GPPF flight hardware performance; (c) Tested the effect of near weightlessness on the light output of FOTRAN stimulus bulbs during a series of parabolic flights on the KC-135 aircraft. Such measurements were needed to determine quantitatively what increased light output would be expected during FOTRAN tests in Spacelab.

Publications

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MICROGRAVITATIONAL EFFECTS ON CHROMOSOME BEHAVIOR

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Description of Research

The long-term goal of this research project is to assess and quantitate the potential genetic risk of the space environment to man. To achieve this goal we are: (1) studying at the basic level the molecular mechanisms involved in the correct separation of chromosomes and, (2) constructing a microbiological system to be used as a model for detection of genetic alterations during a spaceflight experiment inside Spacelab.

The focus of the experiments conducted during the past year has been the molecular characterization of the cloned yeast cell-division-cycle *CDC6* gene whose product is involved in correct chromosome segregation during mitosis. This gene encodes a protein of approximately 58,000 daltons which could represent a potential target for the effect of microgravity and/or cosmic radiation in eukaryotic cells. We have shown that the expression of this gene is regulated throughout the cell cycle and that ultraviolet radiation has an inductive effect on it.

The phenotype of the mutant carrying the temperature-sensitive *cdc6-1* mutation has been analyzed extensively by Transverse Alternating Field Electrophoresis (TAFE), a technique that allows the separation of intact chromosomes in yeast. During these experiments we have observed that cells carrying the mutation undergo chromosome loss when shifted to the maximum permissive temperature of 30°C. These cells grow very slowly into small colonies and eventually give rise to large colonies that revert to a normal growth rate. In some cases we have demonstrated that this is correlated with the acquisition of a second copy of the missing chromosome, therefore showing restitution of diploidy. In addition, during this analysis we have demonstrated that the mutant shows complex chromosomal aberrations which are possibly due to DNA breaks. This could represent a further model to understand chromosomal aberrations observed in spaceflight samples of plant root tip cells.

Accomplishments

(1) ***The molecular analysis of chromosomal aberrations in the cdc6-1 mutant by the state-of-the-art TAFE technique.***

(2) Characterization of the regulation of the *CDC6* gene product.

(3) Completion of the biocompatibility tests and baselining of the new flight hardware built by NASA Ames Research Center.

Significance of the Accomplishments

In order to understand why exposure to the space environment affects chromosome structure and segregation during cell division, we must first understand how chromosomes behave in normal conditions. To this end, it is of utmost significance to isolate and characterize those gene products that are responsible for the correct succession of events leading to the duplication and then the division of chromosomes. With the first

accomplishment we have provided evidence for a specific genetic defect leading to chromosome aberrations. In addition, alteration of the recombination frequency between genes can lead to the expression of hidden mutations and, ultimately, to cancer. The second accomplishment, which relates to the characterization of a protein directly involved in the mechanism of chromosome segregation and recombination, is of significance to the study of the potential cellular targets of the spaceflight environment in eukaryotic organisms.

This significance, together with the modelistic value of the yeast system and its experimental genetic manipulability, provides an ideal system to study large numbers of genetic events in the Spacelab facility with reduced handling, biohazard, and cost.

Publications

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THE ROLE OF CALCIUM IN GRAVITY SENSING

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Description of Research

The overall goal of this research is to determine how gravity is sensed by plants, and how this information is transduced into changes in the growth rates of plant organs leading to gravicurvature. There is convincing evidence that calcium is required in two steps: at the site of perception of gravity and at the site of the unequal growth rates on the two sides of an organ which lead to curvature. A current hypothesis states that a lateral redistribution of calcium occurs at both sites in horizontal stems and roots, and is essential for the response. At the site of perception, the calcium redistribution is believed to be directly involved in the redistribution of the actual growth-controlling hormone. At the site of curvature, it has been suggested that an accumulation of calcium in the walls of the future concave side inhibits wall extension. Direct evidence for both of these ideas is limited. Our objective is to provide evidence as to whether changes in extracellular calcium are directly involved in either the perception or the reaction stages of gravitropism.

Our research has been directed along three main lines. The first is a study of the concentration of free extracellular calcium on the upper vs. lower sides of corn root tips, where the graviperception occurs. Depending upon the theory, a higher, lower, or equal concentration of calcium would be expected on the upper side.

The second project has been to measure the total calcium on the upper vs. the lower sides of stem and coleoptile tissues. Previous studies have suggested that a redistribution of calcium occurs during the gravitropic curvature, with the calcium moving towards the slower growing upper side. However, these previous studies did not actually measure total calcium. As a result there have been serious questions about these results.

The third project has been to determine the role of wall-bound calcium in regulating the ability of cell walls to undergo extension. One widely held hypothesis involves calcium crosslinks between pectic chains serving to stiffen the walls; it is suggested that the redistribution of calcium during gravicurvature is responsible for the difference in wall extensibility on the two sides by altering the numbers of calcium crosslinks on the two sides. If this hypothesis is correct, removal of wall calcium should increase the extensibility of the walls. This hypothesis has been put to a direct test. Associated with this has been a study of the relationship between wall-bound and free apoplastic calcium. Cell wall loosening is believed to occur when cells excrete protons into the apoplast. The resulting acidification of the apoplast should displace wall calcium, raising the free calcium level. However, the amount that the free calcium concentration increases depends upon a complex interaction between pH and the amount of free and bound calcium. This relationship has not previously been analyzed.

Accomplishments

(1) The calcium microelectrode system has been developed, and the first measurements of the extracellular calcium concentrations at the tip of corn roots have been

made. The calcium activity is 2-6 mM. When placed horizontally, the calcium concentration on the top side appears to show a small decline.

(2) The concentration of total calcium has been determined for both vertical and horizontal stems of sunflower and pea and for coleoptiles of corn. In each case the concentrations on the upper and lower halves of the horizontal organs have been compared with that of vertical organs. In addition, the distribution of calcium down a stem or coleoptile has been determined, as has the amount in the epidermal layers vs. the cortical tissues. In all three organs, the calcium concentration increases with distance down the stem; i.e., older cells have more calcium. With both stems, the epidermal layers contain more calcium than the cortical cells. ***In all three organs there is no difference in calcium between upper and lower sides, or between epidermal layers of upper and lower regions.***

(3) In soybean hypocotyls, removal of wall calcium with either protons or chelators results in a decrease in wall strength, as measured with the Instron technique, but only after a considerable amount of the wall calcium has been removed. ***When frozen-thawed walls are placed under tension, they undergo extension in response to acid. It has been shown that this is not due to solubilization of calcium from the walls,*** since removal of wall calcium with the calcium chelator Quin 2 causes no comparable wall extension.

(4) It has been shown that the wall calcium is lower than predicted, assuming an apoplastic calcium concentration of 1 mM and a pH of 5.5. The relationship between wall-bound calcium and free calcium, as a function of pH, is currently being investigated.

Significance of the Accomplishments

Finding #1: We are now ready to make the difficult measurements of the concentration of free calcium at the gravisensing region of the root. During the next year we should be in a position to determine whether gradients of extracellular calcium exist in this region.

Finding #2: Our data demonstrate directly that redistribution of total calcium does not occur during gravitropism, either with stems or coleoptiles. We cannot eliminate the possibility that redistribution of calcium within the walls occurs, or that redistribution between the wall and the cytoplasm might take place.

Finding #3: Our data demonstrate that calcium crosslinks do not form significant stiffening agents in soybean hypocotyl cell walls, and that breakage of these links with protons is not the cause of cell wall loosening. They show that theories of gravitropism based on changes in wall calcium are not viable.

Finding #4: This preliminary information indicates that cell walls contain far less calcium than can be predicted on the basis of the expected apoplastic calcium and pH levels. The reasons for this discrepancy are not known, but could be important with regard to the possible role of free apoplastic calcium as a regulator of cell elongation.

Publications

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MECHANISM OF DIFFERENTIAL GROWTH DURING STEM GRAVITROPISM

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Description of Research

We are using gravity as an experimental tool to probe the basic mechanisms underlying plant cell expansion and its control. When plants are placed in a horizontal position, their stems typically grow upright and their roots grow downwards. This gravitropic bending occurs because of a growth redistribution on the upper and lower sides of the organ. We are using young cucumber seedlings as a model system to investigate the mechanism of gravitropism because they show a very vigorous response: after a lag of about 10 min the upper stem surface ceases growth entirely whereas the lower surface doubles or triples its expansion rate. Since the stem diameter is only 1.5 mm, this means that cells very close to one another respond to gravity in a large and opposite manner. Elucidation of this rapid growth response will likely tell us a great deal about how plant cells control their growth rates.

When intracellular pressure, or turgor pressure, was measured with the pressure probe, we found only a negligible, passive change on the two sides of the stem. This demonstrated that cell expansion was not modulated by altering cell turgor or other related hydraulic properties of the growing cells. By use of stress-relaxation analysis with growing tissues and with isolated cell walls, we found that the wall on the upper stem was not physically stiffened during the period when its growth was inhibited. Rather, the active process of wall loosening was inhibited. At present the nature of this loosening process is uncertain. We are focusing on the working hypothesis that enzymatic modification of the wall regulates wall loosening, and that the activity of these hypothetical enzymes is regulated during the gravitropic response, perhaps through changes in the ionic (Ca^{2+}, H^{+}) environment of the wall or through some covalent modification. In the past year we have focused primarily on the possible role of wall calcium.

Accomplishments

(1) The calcium-binding capacity of walls isolated from cucumber stems was measured. The walls exhibited one major binding site with a binding constant of about 0.8 mM and a binding capacity of about 200 μmoles per g dry weight.

(2) Under the growing conditions we use, cucumber stems have a total calcium content of 680 μmoles per g fresh weight. ***Bulk calcium is not significantly redistributed during the early phases of gravitropic bending.***

(3) ***External calcium stimulates cucumber stem growth.*** As external added calcium was increased up to 10 mM, the growth of the seedlings was stimulated. Above this value, calcium became inhibitory, but this was likely an osmotic effect.

(4) External calcium raises the level of free calcium in the walls of the cucumber stem. Free calcium was measured by ion-specific microelectrodes. With no calcium added to the rooting medium, the free calcium averaged 0.25 mM. Upon external addition of 10

or 100 mM $CaCl_2$, the free calcium increased to 50-70% of the external concentration. ***Nevertheless, these high-calcium plants exhibited a very strong gravitropic response.***

(5) Adding calcium to isolated cucumber cell walls had little effect on their mechanical properties, as measured by Instron and stress-relaxation analyses.

(6) A device for optical detection of wall pH was designed and constructed. It consists of a fluorescence microscope coupled to a scanning monochromator-photomultiplier tube and a microcomputer for data acquisition from the optical detector. With the use of fluorescent pH indicators, this instrument will be used to measure changes in wall pH during gravitropic bending of plant organs.

Significance of the Accomplishments

These results tells us about the state and significance of calcium in the cell wall. Findings #3 and #5 indicate that at physiologically meaningful levels ***calcium does not inhibit cell expansion or rigidify the cell wall in cucumber.*** This conclusion is quite different from the conventional view that calcium at mM concentrations inhibits plant growth, e.g., in oat coleoptiles and pea epicotyls.

There is no indication that bulk calcium is redistributed in cucumber during gravitropism (Finding #2), or that its concentration is regulated (Finding #4), or that extraordinarily high levels of extracellular calcium interfere with the normal gravitropic response (Finding #4). These results provide additional evidence against the hypothesis that changes in extracellular calcium play an important role in the transduction of gravitropism in cucumber stems.

Publications

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GRAVITY DETECTION MECHANISM IN A SINGLE CELL, THE *PHYCOMYCES* SPORANGIOPHORE

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Description of Research

The complexity of multicellular plant tissues which sense, coordinate, and respond to gravity-induced signals limits direct investigation of the various components of gravitropism and relegates data interpretation to the realm of correlation. Considering the appealing possibility that stretch-activated ion channels (SACs) evolved early and were modified to serve as gravity sensors in a wide variety of organisms where orientation to Earth was important, research of such a mechanism in a lower, simplified single cell system has the potential of yielding more direct information about the relationship between SACs and gravity detection, as well as providing data for evolutionary interpretation of the development of gravity detection in plants. The gravitropic response of the upright single-celled reproductive sporangiophore of the terrestrial fungus, *Phycomyces*, is documented in both Earth and microgravity conditions and its easy culture/handling make it highly suitable for investigation of gravity detection.

SACs are detected by patch-clamp analysis. Such channels have been observed in higher plant cells of non-gravitropic tobacco suspension culture cells and tentatively in putative columella cells from corn root caps. The difficulty of such analysis is that it requires the cell wall to be removed to access the plasmalemma; such membranes are stripped of protein with time exposure to wall digestive enzymes, which renders it difficult to patch consistently. *Phycomyces* sporangiophores, having a chitinous cell wall, are large (2-4 cm long in early stages) and produce membrane vesicles when the sporangiophore tip is excised and the cytoplasm is extruded. These vesicles are highly suitable for patch-clamp analyses.

Focus during the last year has been on examining *Phycomyces* sporangiophores for evidence of SACs by patch-clamp and physiological methods.

Accomplishments

(1) ***Patch-clamp analysis has revealed several channels in isolated membrane vesicles, one of which is a low conductance, voltage-independent, weakly selective cation channel.*** The presence of channels in this airborne single cell attests to the nature of the plasmalemma as communicator between cell and cell wall and that the cell wall can serve as a compartment in which external ions can diffuse from the ground-associated mycelial wall component.

(2) The possibility that the gravitropic system of *Phycomyces* is unique and holds nothing in common with that of higher plants exists, particularly in light of the fact that the phototropic receptor system of the *Phycomyces* sporangiophore is different from that of higher plants. To examine the gravitropic nature of *Phycomyces* relative to the complex tissues of root and shoot, we considered the effect of the heavy metal, gadolinium. Gadolinium, at low concentrations, has been shown to inhibit specifically gravitropism of higher plant roots and shoots. Of exceeding interest to proponents of the role of SACs in gravity detection is that gadolinium has also been shown to specifically inhibit SACs in

Xenopus oocytes at concentrations as low as 10 μM. ***Through application of gadolinium chloride in the growth media of Phycomyces, the resulting sporangiophores arising from the mycelial mat are gravitropically inhibited without reduction of symmetric vertical growth rate*** at concentrations of 5 μM to 0.5 μM. This is comparable to the effect on higher plants and SACs in oocytes.

(3) Sporangiophores arising from mycelium grown on media containing 250 μM gadolinium chloride were severely retarded. When a mycelial patch from this culture is transferred to media without gadolinum, the first sporangiophores produced show retarded elongation and no gravitropism, while sporangiophores produced later at a longer radius from the gadolinium-treated mycelial source show normal growth but growth which is not or is only weakly gravitropic. This suggests that gadolinum can diffuse from the mycelial cells to the upright sporangiophore most likely via the cell walls.

Significance of the Accomplishments

That *Phycomyces* has ion channels in membrane vesicles provides hope that indeed SACs may be found in the sporangiophore membrane. Their linear location in the cell membrane and their density will affect their detection by patch-clamp of membrane vesicles. That sporangiophores are exceedingly sensitive to gadolinium-induced inhibition of gravitropism lends credence to the possibility that the gravitropic mechanism has similarities to that of higher plant gravitropic responsive tissues.

Publications

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ROLE OF CALCIUM IN SIGNAL TRANSDUCTION IN ROOT GRAVITROPISM

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Description of Research

This research is directed toward understanding the influence of gravity on plant growth — in particular how roots become oriented with respect to gravity (gravitropism). The detection of gravity occurs at the tip of the root while adjustments in growth rate occur in the growing region about 0.5 cm behind the tip. There is evidence that gravity-induced redistribution of calcium within the tip links gravidetection to asymmetric distribution of the growth-inhibiting hormone, auxin, in the growing region of the root. We are interested in the pathway of movement of signal from the cap to the elongation zone and in the involvement of root cap calcium and calmodulin (a small protein thought to mediate calcium action in many cases) in the establishment of the growth asymmetry that leads to root curvature.

Our research has centered on the following: (1) preparation of protoplasts from cells of the elongation zone and cap of maize roots and investigations of methods of assaying their calcium and calmodulin content; one goal of this research is to determine whether there are differences in cytoplasmic calcium levels in the two cell types and whether or not treatments known to affect gravitropic sensitivity also affect cell calcium; (2) experiments involving surgical alteration of potential transport pathways between the root cap and elongation zone as a means of testing the route of movement of the gravitropic effector; in these experiments we remove entire rings of cortical tissue from around the root at various positions between the cap and elongation zone and determine the effect on gravitropism; and (3) tests of the basis of the effect of ethylene (a plant hormone known to modify root growth) on root gravitropism; treatment of roots with ethylene leads to exaggerated gravitropic curvature.

Accomplishments

(1) We have isolated protoplasts from both the root cap and the elongation zone of maize roots. As shown in Figure 1, protoplasts from the two cell types are different (cap protoplasts are smaller, not spherical, and have more dense cytoplasm). We are testing the uptake and calcium-reporting ability of the following calcium indicators: chlorotetracycline (CTC), indo-1, fura-2, and fluo-3, using both a spectrofluorometer and (in the case of fura-2) single cell quantitative fluorescence microscopy. Preliminary results indicate that ***loading of the indicator dyes is not pH sensitive in root protoplasts but that fura-2 and indo-1 can be loaded as the acetyl ester. It appears that the cytoplasmic free calcium level is much higher in protoplasts from the elongation zone than in protoplasts from the root cap.***

(2) One model for root gravitropism suggests that the gravitropic effector moves from the root cap into the elongation zone through cells of the cortex. ***When we remove a narrow (0.5 to 1 mm) ring of cortical tissue from the root within the apical portion of the elongation zone, we find that gravitropic curvature occurs apical to the girdle but not behind it (Figure 2).*** Related work shows that the gravitropic signal ***will*** pass a narrow circumscribing score line (incision, no tissue

removed) within the elongation zone, and in some cases will pass across a girdled zone which is filled with a diffusion medium such as agar.

(3) Maize root gravitropism is greatly exaggerated in the presence of ethylene (e.g., 100 ppm). The roots continue to curve past 90 degrees and sometimes make a complete loop. We investigated two potential explanations of this phenomenon: (1) ethylene prevents the adaptation of roots to inhibitory levels of auxin on the lower side, hence prolonging growth asymmetry and curvature, and (2) ethylene prevents adaptation of the gravireceptor apparatus to gravistimulation, thus prolonging the effect of gravistimulation. ***We find that ethylene does not prolong the inhibitory effect of applied auxin, indicating that the effect of ethylene on gravicurvature is not exerted by modification of the time course of adaptation to an auxin gradient. On the other hand we find that preadaptation of roots to gravistimulation (by a series of unilateral gravity exposures each too short to induce curvature) reduces or eliminates the ethylene effect.***

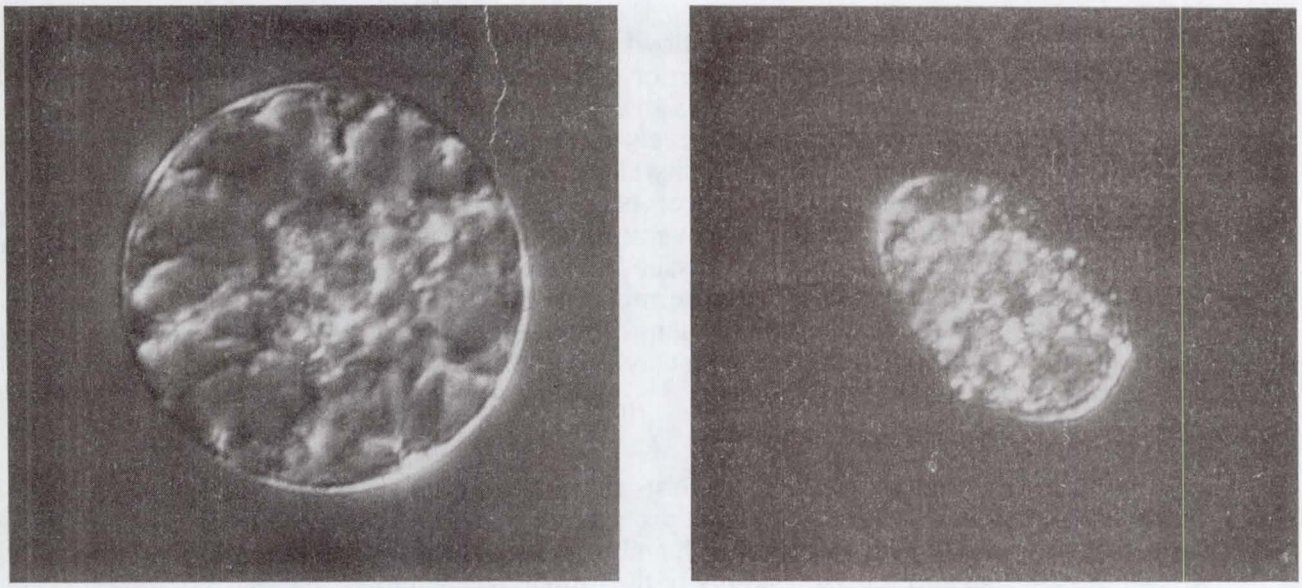

Figure 1. Protoplasts prepared from the elongation zone and the cap of primary roots of 3-day-old seedlings of the maize cultivar Merit. Left: protoplast from elongation zone (X1428). Right: protoplast from root cap (X1540). Note differences in size, shape, and cytoplasmic density. The particular cell type from which the protoplasts are derived is not yet determined.

GRAVITROPISM IN ROOTS OF MAIZE

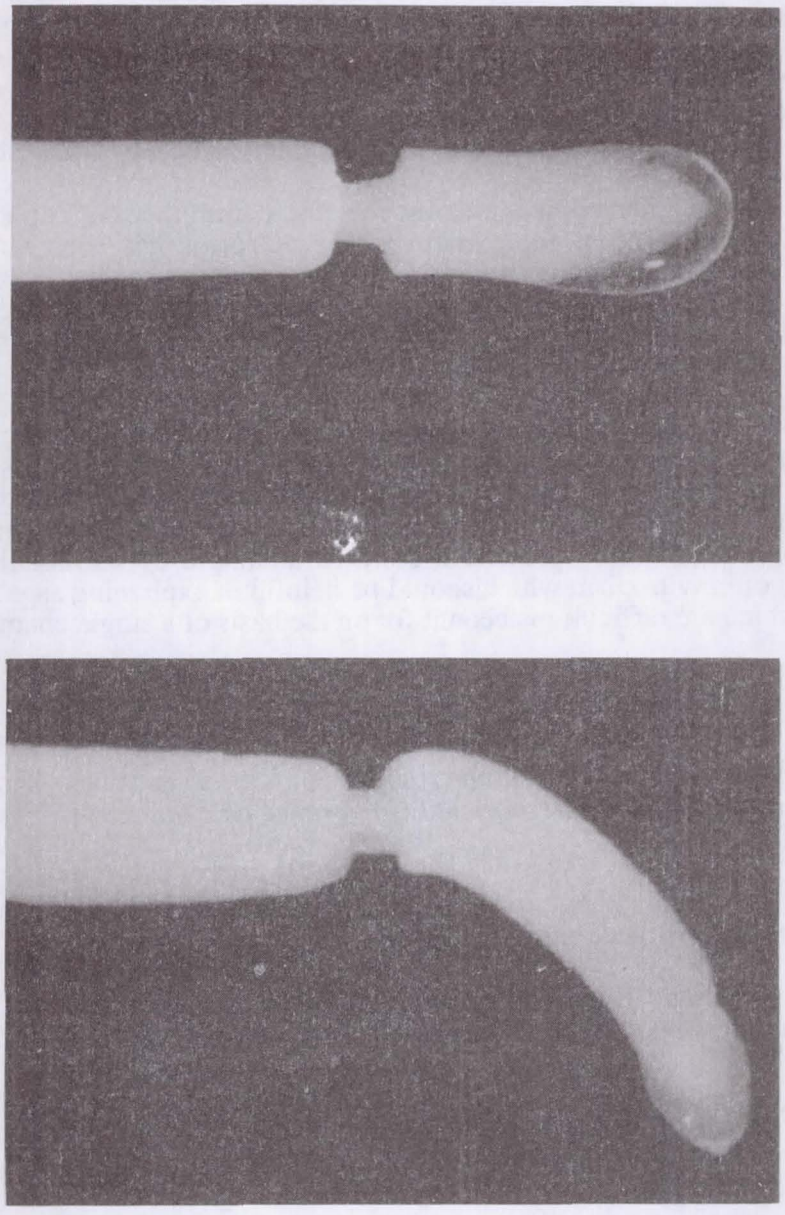

Figure 2. Gravitropism in primary roots of 3-day-old seedlings of the maize cultivar Merit from which portions of the cortex have been surgically removed. Top: a ring of cortex about 0.5 mm wide has been removed from the elongation zone and the root oriented horizontally (zero time). Bottom: gravitropic curvature 12 h after gravistimulation of the "girdled" root. Note that there is gravitropic curvature apical but not basipetal to the girdle. The results indicate that the cortex is necessary for basipetal movement of the gravitropic signal.

Significance of the Accomplishments

Finding #1: The ability to isolate healthy protoplasts from the root cap and elongation zone of maize roots and to load them with calcium indicators will allow us to compare the calcium balance of cells from both the detection and response ends of the gravitropism mechanism. Calcium has been proposed to mediate transduction of the gravitropic signal. The development of these methods will enable us to test the effects of factors known to modify gravitropic sensitivity (light, abscisic acid) on the calcium balance of the gravidetecting cells of the cap. The preliminary observation that the cells of the root cap have unusually low cytoplasmic levels of calcium is especially interesting in this regard.

Finding #2: The gravitropic signal moving back from the root cap cannot pass across a region of the elongation zone where a ring of cortex has been removed. This is an important observation since there is disagreement concerning the route of signal movement from cap to root. Our data suggest that the signal must move through the cortex and/or epidermis. These data are consistent with our model of gravitropic signal transmission in roots and they are important to evaluating alternative models.

Finding #3: The ability of ethylene to cause exaggerated gravitropic curvature in maize roots is the result of retardation of adaptation to the gravistimulus. This is the first evidence for a hormonal effect on the timing of adaptation to gravistimulation and it provides a potential means of investigating the biochemical basis of adaptation to gravistimulation. These observations also provide a concrete example of hormonal interaction in the regulation of gravitropism which should be helpful in explaining aspects of graviresponse kinetics which are difficult to account for on the basis of a single chemical mediator of the response.

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PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR PROCESSES ASSOCIATED WITH GRAVITROPISM IN ROOTS OF MAIZE

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Description of Research

On Earth roots typically respond to gravity by growing downward. Our research focuses on the physiological, biochemical, and molecular steps involved with transducing the gravity stimulus into a developmental response.

During the past year our efforts have been divided into two areas. First, we are pursuing an earlier finding which suggested that mRNA synthesis within the root cap is required in order for a root to respond to gravity. Secondly, we are studying particular events hypothesized to be involved in transduction of the gravity stimulus.

For the first portion we are using a variety of corn in which root orientation is dependent not only on gravity, but on light as well. When seeds of this variety are germinated in darkness the roots orient parallel to the Earth's surface. However, when the roots are briefly illuminated, the direction of root growth changes from parallel to perpendicular to the Earth's surface. It is assumed that light removes some block in the processing of the gravity signal. Earlier we showed that light affects the synthesis of many proteins. During this most recent period we found that these light-induced changes in proteins are modulated, at least in part, by changes in the levels of mRNA. Moreover, the magnitude of the changes in mRNA levels is greater than the changes in the proteins for which they code. Originally we had hoped that the changes in mRNAs would be restricted to only a few species, but instead we found that many mRNAs are affected by light, with most increasing in their levels as a result of illumination, although some decreased as well. In an effort to focus on specific mRNAs within the root cap (in which gravity transduction occurs), we carried out *in situ* hybridization looking for changes in specific messages. We have concentrated on the messages for two proteins in particular, phytochrome and calmodulin. Our reasoning in looking for the messages of these two proteins is that both have been hypothesized to have a role in gravity transduction. With regard to the message for calmodulin, we were unable to find a change in either message location or amount following illumination, suggesting that light is not affecting gravitropism via calmodulin. For phytochrome we have found complicated changes in the message pattern following illumination. Moreover, these changes suggest that the effects of light may extend beyond simply influencing events within the cap. We are continuing to try and correlate light-induced changes in phytochrome expression within root tissue with gravity transduction.

The second portion of our effort has been to investigate specific steps involved in gravity signal transduction. Currently, it is hypothesized that processing of the gravity signal in the cap involves the phosphorylation of proteins by kinases. Unfortunately, very little is known about plant kinases. Indeed, none has been isolated, nor has the message for any been sequenced. Towards understanding more about kinases, and if they are present in the cap, we have screened a maize root cDNA library using synthetic oligomers homologous for the conserved regions of kinases in microorganisms and animals. This effort has resulted in the positive identification of several kinase clones. One clone is for a cyclic AMP-like kinase. This kinase does not show specificity for the root cap, but rather is

found in both the cap and in adjacent root tissue. The challenge ahead is now to determine whether this kinase may have a role in transducing gravity.

Accomplishments

The major findings from these studies are:

(1) ***Light affects the levels of many distinct mRNAs located within the root cap. In most instances light enhances message levels, but in some cases mRNAs decrease as a result of illumination.***

(2) ***Light effects on calmodulin mRNA are not confined to the root cap, but rather occur throughout the tip of the root. Therefore, it is unlikely that light influences root gravitropism via alterations in calmodulin.***

(3) ***Kinase mRNAs are present in the root cap. This observation supports the hypothesis that kinases could have a role in gravity signal transduction in roots.***

Significance of the Accomplishments

In order to understand the steps associated with gravity transduction, we have used a variety of maize in which light is required for the roots to respond completely to gravity. We hoped to find that only a few events were stimulated by light. Our results, however, show that many proteins and their corresponding messages are affected by light. Thus, it appears that light-mediated root gravitropism may involve many proteins, and their messages, and not just a single species. In other words, there does not seem to be a specific gravity transduction protein. However, because certain proteins were hypothesized to be involved with gravity, we looked specifically at these (calmodulin and phytochrome). We hoped to see whether light preferentially affected the levels or distribution of these proteins or their messages. Our *in situ* analysis did not reveal a localized increase in these proteins, thus confirming our earlier conclusions that light effects are more general and not confined to only a few species. By documenting the presence of kinase messages in the root cap, our results support the possibility of the involvement of phosphorylating enzymes in transducing the gravity stimulus.

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THE EFFECTS OF TEMPERATURE AND HYPERGRAVITY ON THE GROWTH RATE AND CIRCADIAN RHYTHMS IN *NEUROSPORA CRASSA*

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Description of Research

Diurnal oscillations in physiological function, behavior, and morphology observed in eukaryotic organisms: are they controlled by a central endogenous pacemaker(s), i.e., circadian clock(s), or are they driven by environmental time cues? This is the central question being addressed in our project. Circadian research is characterized by the observation of organisms in conditions where environmental oscillations are minimized. Light, a major modulator of biological oscillations, is usually continuously on or off during experimentation; temperature is held constant, and other diurnal variations such as noise and social interaction are usually severely attenuated. The control of some environmental oscillations is much more formidable; alterations in humidity and barometric pressure are usually not under experimental control, and oscillations such as the gravitational pull of solar and other planetary bodies are difficult, if not impossible, to eliminate on the surface of the Earth.

Two opposing hypotheses, which have been proposed to explain the generation of biological diurnal oscillations, have been debated in the circadian literature over the past 30 or 40 years. One hypothesis states that these oscillations are passive responses to a fluctuating environment. The rhythms, then, are thought to be derived from an *exogenous* rhythmic influence (such as solar illumination), which imposes temporal information and control over a passively responding system. The second hypothesis, which purports *endogenous* control of diurnal oscillations, implies that within the organism there exists an active timekeeping device that is capable of approximating the 24 hour solar day, without input from the environment.

Our model organism is the filamentous fungus, *Neurospora crassa*. This bread mold expresses a diurnal variation in conidiation (asexual spore formation). This oscillation can be seen visually as a daily alteration between a low growing surface mycelium and a surface mycelial growth with aerial hyphae which pinch off to form conidia (asexual spores). The mycelium that contains aerial hyphae is clearly seen as a band when grown on media in petri dishes or long cylindrical tubes, known as race tubes. In constant conditions the cycle of banding is repeated approximately once every 22 hours (i.e., the rhythm is circadian).

In 1983, STS-9 carried an experiment in which 24 race tubes inoculated with the BND strain of *Neurospora* were exposed to the microgravity environment of space. The results demonstrated that the rhythm could persist in space; however, there was an increase in the period of the oscillation and the variability of the growth rate. The rhythm of conidiation was also damped in one-quarter of the flight tubes. On L+7 days, the tubes were exposed to light during a marking procedure. The light pulse presumably reinstated a robust conidiation rhythm in the damped tubes. The rhythm remained normal for the remainder of the flight.

Since the flight of STS-9, results from ground-based experiments have suggested that the aberrant effects of the conidiation rhythm observed from that flight may not have been due to

the microgravity environment of space but, rather, to the hypergravity of launch. Acute hypergravity via centrifugation can damp the circadian oscillation of conidiation in *Neurospora*, and chronic hypergravity can prolong the period of the rhythm. Simulated microgravity, via the use of a slow-rotating clinostat, does not simulate the increased period length of conidiation, but can diminish the amplitude of the circadian rhythm of conidiation to some extent. Altered orientation of the race tubes to gravity can increase the free-running period and attenuate the amplitude of the oscillation. Thus the circadian rhythm of conidiation in *Neurospora* is sensitive to gravitational variations, just as the system is sensitive to alterations in light; however, just as light has been shown to affect but not drive the temporal oscillations of circadian rhythms, gravity may be a modifier of an endogenously driven biological clock, and not an exogenous driver of a passive system. The debate remains unresolved.

On December 1989, STS-32 is scheduled to lift-off for a 10-day mission. The shuttle will carry the mid-deck life science experiment, "Characterization of *Neurospora* Circadian Rhythms (CNCR)." We are cautiously optimistic that this experiment can finally support one of the two hypotheses and determine whether there is an underlying endogenous mechanism that is responsible for temporal oscillations.

Accomplishments

We were notified late in 1988 that the average temperature of the shuttle mid-deck could be increased during flight from approximately 25°C to 30°C or more. While the period of the circadian rhythm of conidiation in *Neurospora* has been reported as being temperature compensated (i.e., having a Q_{10} very close to 1), the permissive temperature for clock functions is nevertheless limited. The literature suggests that the permissive temperature range was approximately 16-36°C for the BND strain of *Neurospora*. We wanted to determine the temperature range that would reliably permit rhythmicity in the BND and BND/CSP strains of *Neurospora* in our hands.

Figure 1 shows the results of rhythmicity at various temperatures. ***The circadian rhythm of conidiation was robust between 25 and 29°C . At temperatures of 20, 30 and 31°C, rhythms were damped and a significant portion of the tubes were arrhythmic, especially at 20°C. Only a few tubes were rhythmic at 34°C and those that were displayed a damped rhythm. No samples at 18°C display rhythmic conidiation.*** The BND/CSP strain appears less sensitive to temperature than the BND strain. This is similar to the reduced sensitivity of BND/CSP to hypergravity. Experiments in progress are designed to determine the effects of fluctuating temperatures over the length of the experiment.

Figure 2 shows the effect of temperature on the growth rate (in mm/hour; Figure 2A) and period (in hours; Figure 2B) of the conidiation rhythm in *Neurospora*. ***As the ambient temperature rose, the growth rate increased, doubling or tripling over the temperature range measured. The addition of Brij (polyoxyethylene 23-lauryl ether) to the media attenuated the increase in growth rate with increasing temperatures. The period, however, was relatively temperature compensated and does not change significantly or consistently over the experimental temperatures.*** Brij did not significantly affect the period or the amplitude of the conidiation rhythm.

CIRCADIAN RHYTHMICITY OF *NEUROSPORA* AT VARIOUS TEMPERATURES

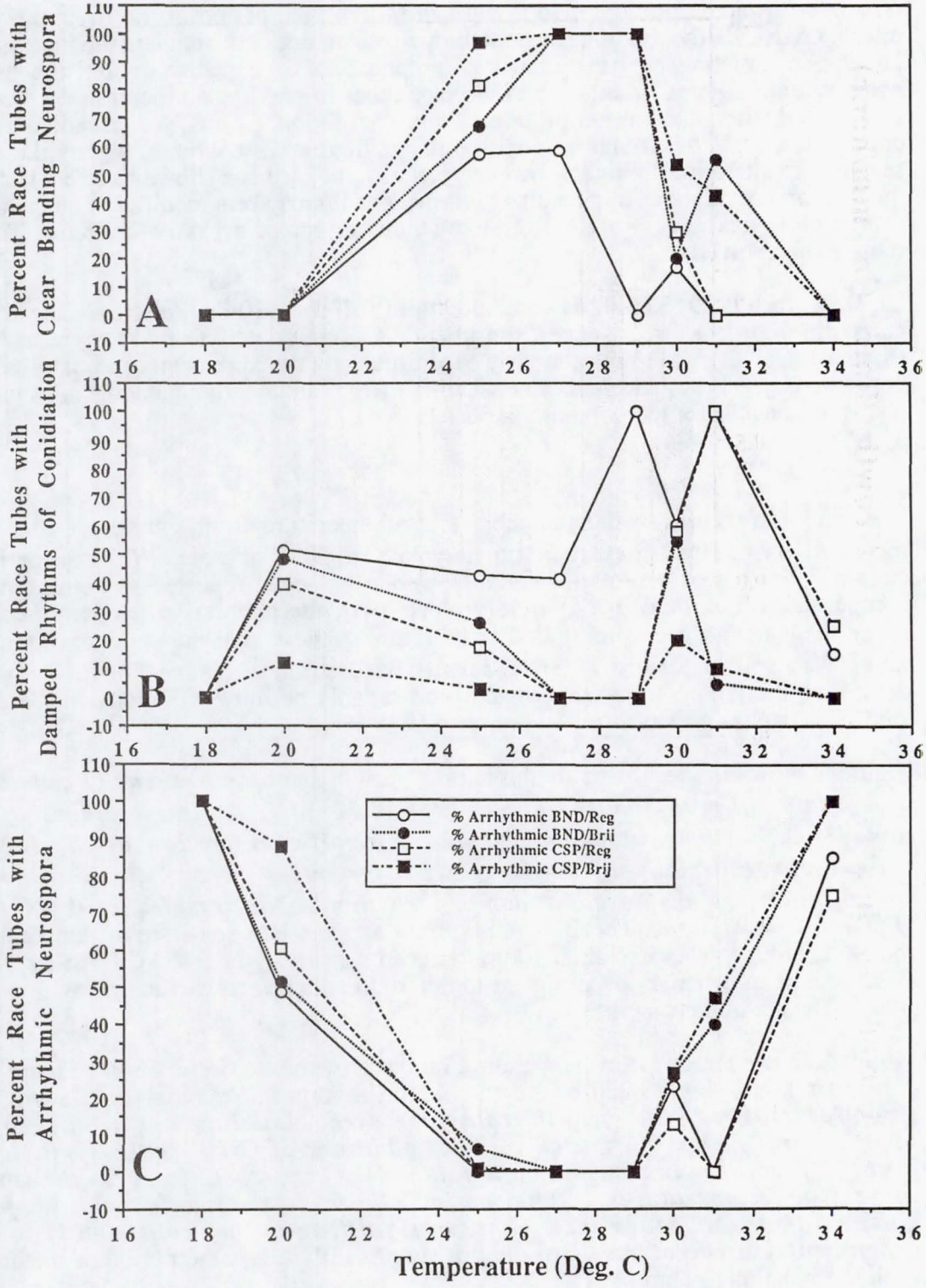

Figure 1. Temperature (°C) versus percent of tubes with clear circadian oscillations (A), damped circadian oscillations (i.e., rhythms with reduced amplitude) (B), and the percent of arrhythmic tubes (i.e., race tubes with no circadian rhythmicity for one cycle or more) (C). Circles represent the BND strain and squares represent the BND/CSP strain of *Neurospora*; open symbols refer to regular media and closed symbols refer to regular media with the addition of Brij (polyoxyethylene 23-lauryl ether).

EFFECTS OF TEMPERATURE/MEDIA ON GROWTH RATE AND CONIDIATION RHYTHM OF *NEUROSPORA*

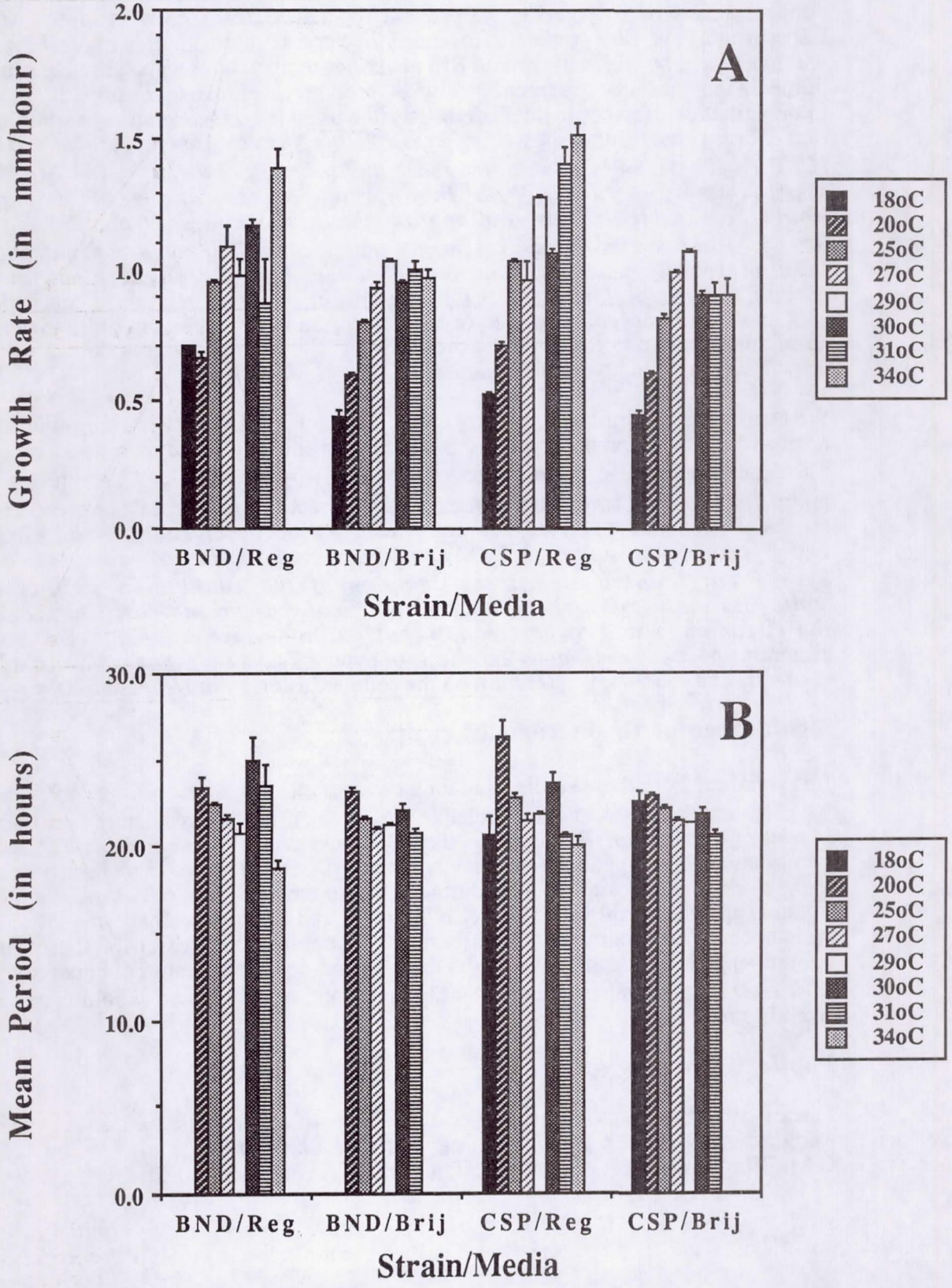

Figure 2. Effects of strain/media and temperature on growth rate of *Neurospora* (A) and the mean conidiation rhythm period (B).

Since the scheduled duration (10 days) of flight STS-32 is longer than the anticipated flight duration (5-7 days), experiments are currently in progress to determine the correct concentrations of Brij needed to prevent the tubes from "growing out" during flight. Experiments have been conducted to determine the maximum duration of *Neurospora* growth within a race tube with and without Brij at various temperatures. The growing surface length of media in a race tube is approximately 280 mm; however, due to the growth necessary prior to the initiation of experimental conditions, the useable surface length is approximately 220 mm. From the results shown in Figure 2, ***the fastest growth rate of 1.5 mm/hour (CSP/Reg at 34°C) would result in tube "grow out" in 146 hours or approximately 6 days. With the normal concentrations of Brij the tubes would be expected to last for approximately 220 hours or about 9 days (up to 34°C)***. Results from these experiments suggest that ***Brij (at a concentration higher than previously used) will have to be added to the flight tubes in order to maintain the experiment for the longer flight duration***. Experiments using 0.5, 1, 2, 3, and 4 times the concentration of Brij are currently in progress in order to determine the correct concentration to insure the prevention of "grow out" without deleterious effects to growth and rhythmicity in this organism.

We conducted our third hypergravity experiment at the University of California, Davis. The results of the temperature experiments were not available prior to this experiment, however. The experimental temperature ranges of the control group (19 to 25°C) and high temperature group (29 to 34.5°C) were too extreme in both directions to delineate between the damping effects of temperature and the damping effects of acute hypergravity. Results from the first two hypergravity studies at UC-Davis, as reported previously, suggest that ***acute hypergravity caused significant damping of the conidiation rhythm at 25°C, while chronic exposure to hypergravity caused an increase in the length of the circadian period of conidiation, but did not cause damping***. Experiments are currently underway to examine the effects of hypergravity (3-6 g) and simulated microgravity (fast clinostat rotation, i.e., 60 rpm) on the cellular histology of *Neurospora*.

Significance of the Accomplishments

It is apparent from these studies that temperatures meeting or exceeding 30°C or below 21-23°C are not compatible with the reliable measurement of the circadian rhythm of conidiation in *Neurospora crassa*. Furthermore, the CSP strain is much more resistant to environmental perturbations of temperature and gravity than BND, and displays an increased likelihood for the greatest data recovery. The addition of Brij to the media not only slows the growth rate, but also appears to aid in the clarity of banding and imposes a small amount of additional resistance to environmental perturbations. Since the conclusion of these temperature experiments, it has been relayed to us that the mid-deck temperatures should not rise above 27-29°C. These temperatures are in the upper optimal range and should insure maximum scientific return.

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GRAVITROPIC RESPONSE MECHANISM IN CEREAL GRASS SHOOTS

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Description of Research

The primary goal of this research is to unravel the gravitropic response mechanism in cereal grass shoots. To achieve this goal, we must first decipher the nature of the gravisensors and how they act to perceive a gravitational signal. Then, we must determine how gravity perception leads to transduction of the signal; that is, when and how hormone asymmetry is established for both of the candidate hormones, native IAA (indole-3-acetic acid) and gibberellins. Finally, it is essential to elucidate how the hormone asymmetry, once achieved, leads to asymmetric growth in an upward-bending pulvinus. Primary components of this unequal growth response mechanism include: differential protein synthesis, sucrose and starch catabolism, beta-D-glucan turnover in the cell walls, and cell wall loosening and synthesis. Our current research focuses on each of these components of the gravity response.

Accomplishments

(1) Graviperception in Cereal Grass Pulvini

We have demonstrated that starch-filled chloroplasts in statenchyma cells of the pulvinus act as the primary gravisensors in the grass pulvinus system. Dark-induced loss of starch in the chloroplasts results in loss of gravitropic response, and reconstitution of starch in these organelles by sucrose feeding results in restoration of the graviresponse in isolated pulvini. Such pulvini do not lose their ability to respond to hormones such as IAA and gibberellins.

(2) Transduction in Cereal Grass Pulvini

(a) ***Pulvini with the node excised show no significant gravi-response over 24 hr. Pulvini excised with the node attached do gravirespond.***

(b) In the vertical orientation, pulvini rapidly take up ^{3}H-IAA, with or without the node. Polar auxin transport (export of label into receiver blocks) is similar in pulvini with or without the node.

(c) ***After gravistimulation, export of label into receiver blocks decreases, with or without the node. The ratio of label in the lower pulvinus half versus that in the upper half increases from 1.0 to 1.5, but only in pulvini with nodes.*** This ratio first significantly differs from 1.0 at 60 min (using 10 min increments) and is maintained at approximately 1.5 for at least 8 hr. Also, movement of label into agar blocks placed atop or below the horizontal pulvinus is asymmetric in gravistimulated pulvini with nodes. This asymmetry is comparable in size and direction to that measured in the pulvini themselves.

(3) Gravity Response Mechanism in the Pulvinus System

(a) Invertase, which catalyzes the hydrolysis of sucrose into D-glucose and D-fructose, changes markedly in response to gravistimulation. ***In lower halves, by 24 hr, its level of activity is 28-fold higher than in pulvini of upright controls; in upper halves, the level of invertase activity only rises 7-fold within the same period.*** The first changes in top/bottom asymmetry in invertases activity in gravistimulated pulvini are seen within 6 hr after beginning of gravistimulation treatment.

(b) Analysis of cell wall components of the pulvinus indicates that gravistimulation causes no changes in relative amounts of pectins, cellulose, arabinoxylan, xyloglucan, and wall protein. But, it does cause a dramatic change in one of the hemicellulose polymers, beta-D-glucan (made up of mixed beta-1,3-glucan and beta-1,4-glucan). ***After 24 hr of gravistimulation, we see a two-fold increase in beta-D-glucan in the lower halves over that in the upper halves. This is the first report of a significant change in a cell wall polysaccharide component elicited by gravity in a monocot shoot system like the cereal grass pulvinus.***

Significance of the Accomplishments

Finding #1: In the absence of any evidence to the contrary, we have now unequivocally established the fact that starch-filled chloroplast statoliths act as the gravisensors in gravistimulated cereal grass pulvini. How they act as gravisensors, leading to transduction of the response, is an open question. They could act as pressure probes to open hormone or ion channels in the plasma membrane. Or, they may possibly play a role in facilitating the establishment of auxin and gibberellin gradients by eliciting enhanced synthesis of the hormones and/or release of the hormones from their stored conjugates.

Finding #2a: We now have a system for separating graviperception from graviresponse in oat pulvini. Pulvini, which contain graviperceiving statoliths, are the site of response. However, isolation of the pulvinus from other tissues (e.g., the node) eliminates the graviresponse. Inclusion of the node with the pulvinus allows the graviresponse to proceed.

Finding #2b: Upon gravistimulation, basipetal transport declines, with or without the node. This indicates that polar auxin transport is linked to pulvinus orientation or graviperception.

Finding #2c: Lateral transport of label in pulvini preloaded with ^{3}H-IAA occurs only in tissues that show a graviresponse. The timing of redistribution is consistent with this interpretation. However, the resulting asymmetry is less than that measured in other tissues and is less than the asymmetry in endogenous levels of free IAA measured in gravistimulated oat pulvini. Apparently, lateral transport of IAA plays a role in gravitropism in oat pulvini but cannot entirely account for the graviresponse.

Finding #3a: The primary significance of the elevated invertase activity seen in bottom and top halves of graviresponding pulvini is that the pool of hexose available for cell wall synthesis is vastly increased. There is a very close parallel between the asymmetry seen in cell elongation and invertase activity from top to bottom of a graviresponding pulvinus. Since the asymmetry in invertase begins to appear within 2 hr after initiation of gravistimulation, we believe that the cell wall synthesis step in the gravitropic response mechanism starts relatively early — within 60 min after cell wall loosening is initiated (this wall loosening occurs close to 60 min after initiation of gravistimulation based on wall extension analyses).

Finding #3b: Beta-D-glucan is now considered to be a prime candidate for the site of action of hormones such as IAA and gibberellins to affect differential cell wall loosening and synthesis in the grass pulvinus system. It is this polysaccharide component of the hemicellulose matrix in the cell wall that changes significantly in response to gravistimulation. We can thus visualize that the hormones regulate turnover of this polysaccharide (by affecting rates of synthesis and degradation of beta-D-glucan) in such a way as to cause wall loosening, which in turn opens up sites in the polymer for new synthesis. The differential thick-thin regions of the walls of pulvinus collenchyma cells seen after gravitropic curvature has occurred provide a structural basis for this contention (Figure 1). Wall loosening in these cells would occur in the thin regions; such regions could also be sites where new cell wall synthesis occurs.

Figure 1. Light micrograph (using microscopy with crossed polarizers) of collenchymatous bundle cap cells of a control (top) and a 48 h gravistimulated (bottom) oat pulvinus. X 135

The working model depicted in Figure 2 provides a conceptual framework for what we believe is happening in the graviresponding cereal grass pulvinus from the time of gravity perception to the last stages of upward bending.

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MODEL FOR GRAVITROPIC RESPONSE MECHANISM IN CEREAL GRASS SHOOTS

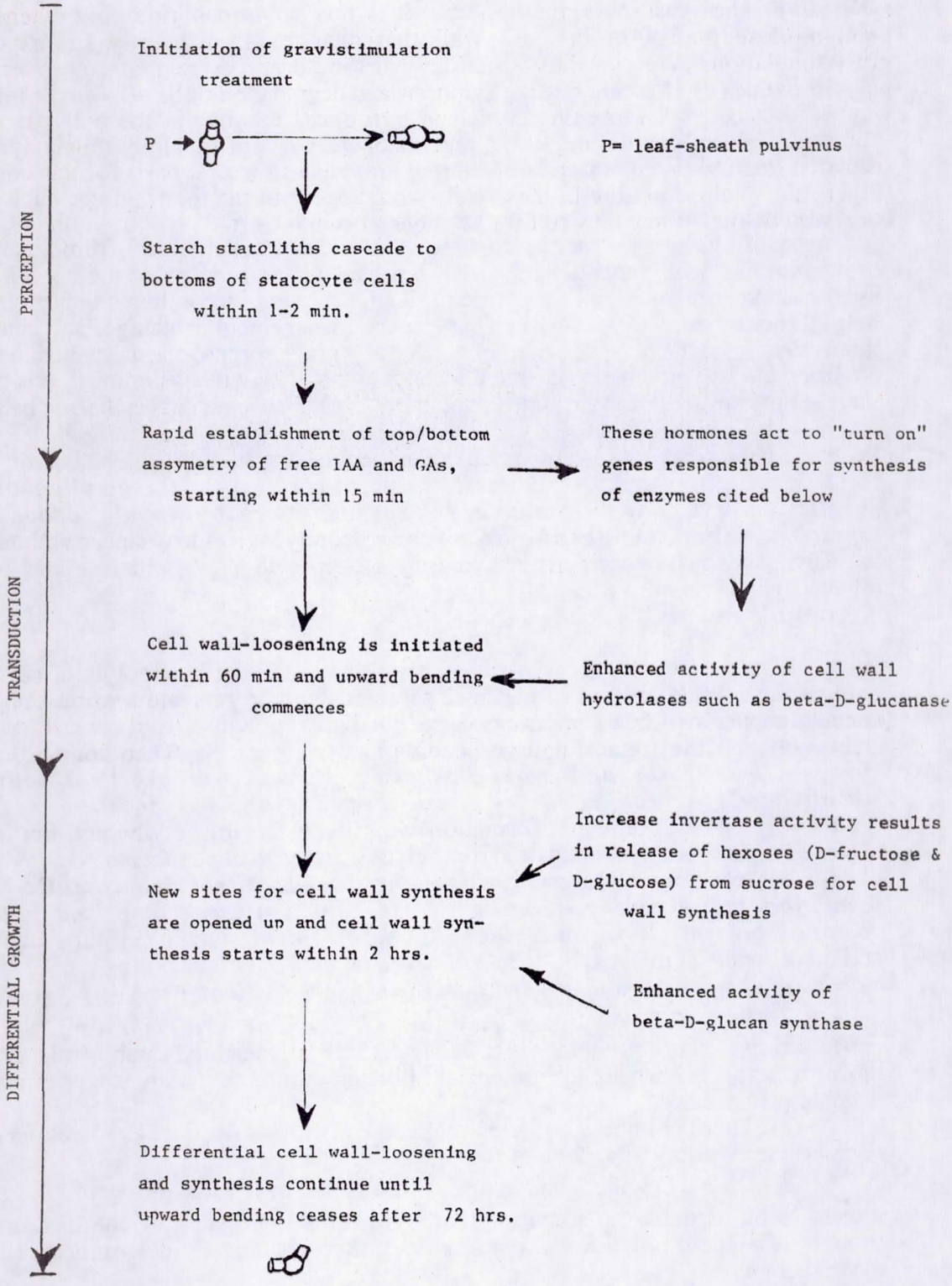

Figure 2. Conceptual framework for graviresponding cereal grass pulvinus following the stages of perception, transduction, and differential growth.

CELL, EMBRYOS AND DEVELOPMENT IN SPACE

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Description of Research

Development entails an orderly progression of cellular events both in terms of time and geometry (dimensional space). However, the study of development from a mechanistic viewpoint has been generally hindered in higher plants because of the complexity that available experimental materials present. Plant cell and tissue cultures *in vitro* have been helpful, but they too frequently present science management challenges and can be labor intensive. From the perspective of implementing gravimorphogenesis studies in the space environment so as to investigate the effects of g-unloading on development, it is becoming increasingly apparent that the simpler and more controlled a system can be, the better.

We have been working towards the development of a system in which somatic embryos form directly from mericarp ("seed") tissue of carrot ***after the germination of the zygotic embryo and without any exposure to exogenously added growth regulators***. Also, other tissues of carrot can respond in a similar manner without growth regulators ever having been invoked.

Accomplishments

Excised zygotic embryos, mericarps ("seeds"), and hypocotyls of seedlings of cultivated carrot cultivars were evaluated for their ability to generate somatic embryos on a semi-solid, hormone-free nutrient medium.

(a) Neither intact embryos nor hypocotyls ever produced somatic embryos. However, ***mericarps and broken zygotic embryos were excellent sources for somatic embryo production (response levels as high as 86%).***

(b) Somatic embryo formation was highest from cotyledons, but was also observed on isolated hypocotyls and root tips of mature zygotic embryos.

(c) ***On media containing unreduced nitrogen, somatic embryo formation led to the generation of vigorous cultures comprised entirely of somatic embryos at various stages of development,*** which in turn proliferated still other somatic embryos.

(d) A medium was devised such that when 1-5 mM NH_4^+ was the sole nitrogen source, ***it led only to a proliferation of globular pro-embryos.*** Sustained subculturing of these proembryos at 2-3 week intervals enabled establishment of highly uniform cultures in which no further development into more mature stages of embryonic development occurred.

(e) These cultures have been maintained, without decline, as morphogenetically competent pro-embryonic globules for over 10 months.

(f) A basal medium containing 1-5 mM NH_4^+ as the sole nitrogen source does not appear to be inductive to somatic proembyro formation. Instead, the medium is best thought of as permissive to the expression of embryogenically determined cells within zygotic embryos.

(g) Once a pro-embryonic culture is established, this medium provides a non-permissive environment to the development and growth of later embryonic stages, but it does allow the continued formation and multiplication of globular somatic proembryos.

(h) The sequence of events leading from excised broken zygotic embryos to the formation of somatic embryos and the maintenance of somatic proembryos has been determined by scanning electron microscopy and histological preparations.

(i) Germination levels from intact zygotic embryos on media with varying levels and ratios of unreduced vs. reduced inorganic nitrogen were determined as well and provided baseline or control data in the type of response obtained from non-wounded material.

Significance of the Accomplishments

This work shows for the first time that a reliable somatic embryogenic response can be elicited from carrot in the absence of any exogenously added growth regulators. The inconvenience and constraints of using suspension cultures from carrot cells that are otherwise demonstrably totipotent are overcome by this system since it: (1) does not require exogenously added growth regulators to initiate or modulate embryogenesis; (2) allows one to observe each somatic embryo throughout its course of development; (3) should not involve difficulties arising from culture-associated or somaclonal variation due to long-term culturing procedures; (4) has a uniform starting explant from year-to-year and laboratory-to-laboratory; and (5) requires little tissue selection or otherwise complicated manipulation.

The findings will permit focussing on what environmental factors (e.g., mineral elements, osmotic pressure, light) modulate the initiation, development, and continued growth of somatic embryos of carrot using the system described above.

Such a system should permit dissection to disclose how environmental effects may vary, depending on the physiological state of determination of the cells being tested. In so doing, it is anticipated that a much more accurate understanding of the physiological and biochemical basis for developmental limitations or effects on somatic embryogenesis such as those involving g unloading in space will be reached.

The work goes far towards making available a truly "model system" that is simple and that can be used by cell biologists, biochemists, molecular biologists, and gravi-morphogeneticists to study the significant mechanisms that make the difference between one morphological result versus another. In this way, "titration" up to a specific switching or control point in somatic embryogenesis can be achieved and studied just as it goes from a "no" to a "yes," or a "yes" to a "no" response level. This will make identifying the biochemical basis of control much more accessible and meaningful.

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PERCEPTION AND TRANSDUCTION OF GRAVITROPISM IN PLANTS

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Description of Research

The objective of this research is to improve our understanding of the sensing and transduction of gravity signals in plants. We use the root gravitropic response of Merit corn seedlings, because the sensing and transductive steps are physically separated from the motor or response region, and because the gravitropic response is highly responsive to red light.

Experimental work in this project has recently been concentrated on the particular role or roles of calcium ions. We have utilized the regulatory action of red light as a means of changing the gravity response system, and employed various calcium modifying chemicals as probes of the calcium participatory roles.

Accomplishments

We have found that the corn root can be made almost nonresponsive to gravity if we lower the calcium concentration in the root with calcium chelator agents. Conversely, ***we can substitute for the red light stimulation of positive gravitropism by applying compounds that are known to stimulate the entry of calcium into plant cells.***

One treatment that apparently increases the calcium concentration in plant cells is abscisic acid. We were pleased to find that ***applications of abscisic acid to the corn root can entirely substitute for the red light stimulation of positive gravitropism.*** This dramatic effect was not associated with any consistent inhibition of root growth rates. ***We confirmed earlier reports that red light causes an increase in abscisic acid content of corn roots.*** We perceive the red light effect, then, as serving in part to alter the calcium availability in the cytoplasm of the sensing or transducing cells.

Another productive line of study has been using the comparison of two types of gravitational responses of the corn roots. In one instance, gravity stimulation causes the roots to bend toward the gravity source; this response is called ortho-gravitropism. In another instance, gravity stimulation causes bending only to the horizontal position; this response is called dia-gravitropism. Red light can convert the diagravitropic response into the orthogravitropic response. Testing these two types of tropisms for their responses to calcium chelators revealed that ***diagravitropism is relatively insensitive to the calcium availability in the root.*** Subsequent experiments on the application of controlled levels of calcium using calcium buffers have revealed that ***there is almost a 100-fold lower requirement for calcium in diagravitropism than in orthogravitropism.*** Thus we feel that calcium can at least partly regulate the nature of the gravity response, holding the switching capability between these two types of gravitational responses.

Significance of the Accomplishments

The unraveling of the processes involved in the sensing and transduction of gravity signals in plants is complicated, and progress in this area will be slow. We feel that the utilization

of calcium regulatory systems provides a new dimension in potential progress for this area of environmental sensing. The linkage of the control by light, the control by calcium, and the distinction of two contrasting types of graviresponses provides exciting possibilities for advancing our understanding of this gravity response.

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DETERMINING THE EFFECTS OF GRAVITATIONAL STRESSES ON LIGNIN FORMATION AND STRUCTURE: A NEW APPROACH

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Description of Research

Lignins are complex phenylpropanoid polymers produced by terrestrial vascular plants. One of their roles is to provide compressive strength to plant cell walls, thereby allowing such structures to stand in upright forms. There is some tentative evidence suggesting that the lignin content of plants can be affected by gravitational loads experienced during growth.

The specific objectives of this research are to determine the effect of gravitational forces on (a) the rate of uptake and binding of specifically labelled lignin precursors; (b) the bonding patterns of lignin *in situ*; (c) the function, formation, and activities of specific peroxidase isozymes responsible for lignin formation; (d) cinnamyl alcohol dehydrogenase activity; and (e) development of vascular tissue.

Accomplishments

(1) ***Incorporation of Labelled Precursors into Lignin, and Bonding Patterns in situ.*** Radiolabelled lignin precursors [2-^{14}C] p-coumaric, ferulic and sinapic acids, feruloyl glucose, feruloyl CoA, p-coumaryl, coniferyl and sinapyl alcohols, coniferin, and phenylalanine were synthesized. The above precursors were supplied to *T. aestivum* L., *L. leucocephala*, *N. tabacum*, and *P. taeda* plants grown hydroponically under aseptic conditions for periods up to two months in duration. These experiments revealed that ***the most efficient incorporation into the lignified portions of leaf, stem, and root tissue was obtained with [2-^{14}C] phenylalanine.***

Next, using [1-^{13}C], [2-^{13}C] and [3-^{13}C] phenylalanine and ferulic acids, we were able to establish the exact bonding patterns of lignified stem and root tissues of the hardwood, *L. leucocephala* (at 1 g). This was obtained by solid state C-13 nuclear magnetic resonance (NMR) analysis of the resulting plant tissue (Figure 1).

Comparative C-14 and C-13 experiments are currently underway to determine effects of gravitational loads (0.05 and 0.5 g).

(2) ***Design of Clinostats for Aseptic, Hydroponic Growth of Plants.*** Several clinostat configurations were tested for the suitability of growing plants hydroponically under aseptic conditions in a vibrationless environment. Since none were problem-free, ***we designed a system to circumvent various problems that we encountered (Figure 2). The apparatus shown is now suitable for hydroponic growth of six plants in an environment where temperature, light intensity, vibrational level, oxygen requirement and sterility can be rigorously controlled.*** This apparatus is now being used for conducting experiments at reduced g levels.

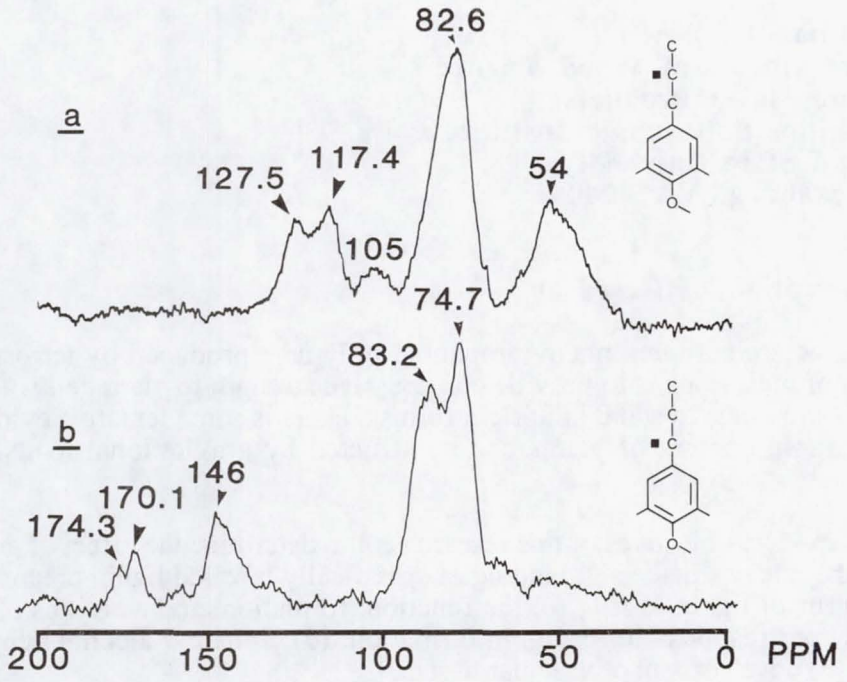

Figure 1. Solid state C-13 NMR difference spectra of *L. leucocephala,* previously administered (a) $2\text{-}^{13}C$ and (b) $3\text{-}^{13}C$ ferulic acids.

(3) ***Function, Formation, and Activities of Specific Peroxidases.*** The isolation of cell wall peroxidases from species such as *Pinus taeda* is rather time-consuming and troublesome. However, since these isozymes are considered to be involved in lignin formation, we have developed a method for their facile isolation. This has been carried out with *P. taeda* suspension cultures, where two peroxidase isozyme forms have been detected. Their role in lignification at different g levels is currently under investigation.

Significance of the Accomplishments

It must be emphasized that progress in determining the effects of gravitational stimuli on plant cell wall polymer deposition necessitated the design of more appropriate clinostats for long term hydroponic growth experiments under aseptic conditions. This has now been completed.

Consequently, experiments on the incorporation of both C-14 and C-13 labelled lignin precursors into the lignified portion of plant tissue are now underway to determine the changes in (a) p-hydroxyphenyl:guaiacyl:syringyl ratios and (b) bonding patterns of lignin as a consequence of gravitational force experienced. Additionally, the activities of key enzymes involved in the lignification process can also be monitored at different g forces. These ongoing studies will provide precise information on how the actual lignin deposition process is altered as a consequence of g force experienced during growth.

These studies will also provide essential information on processes affecting "normal" development of plant cell walls.

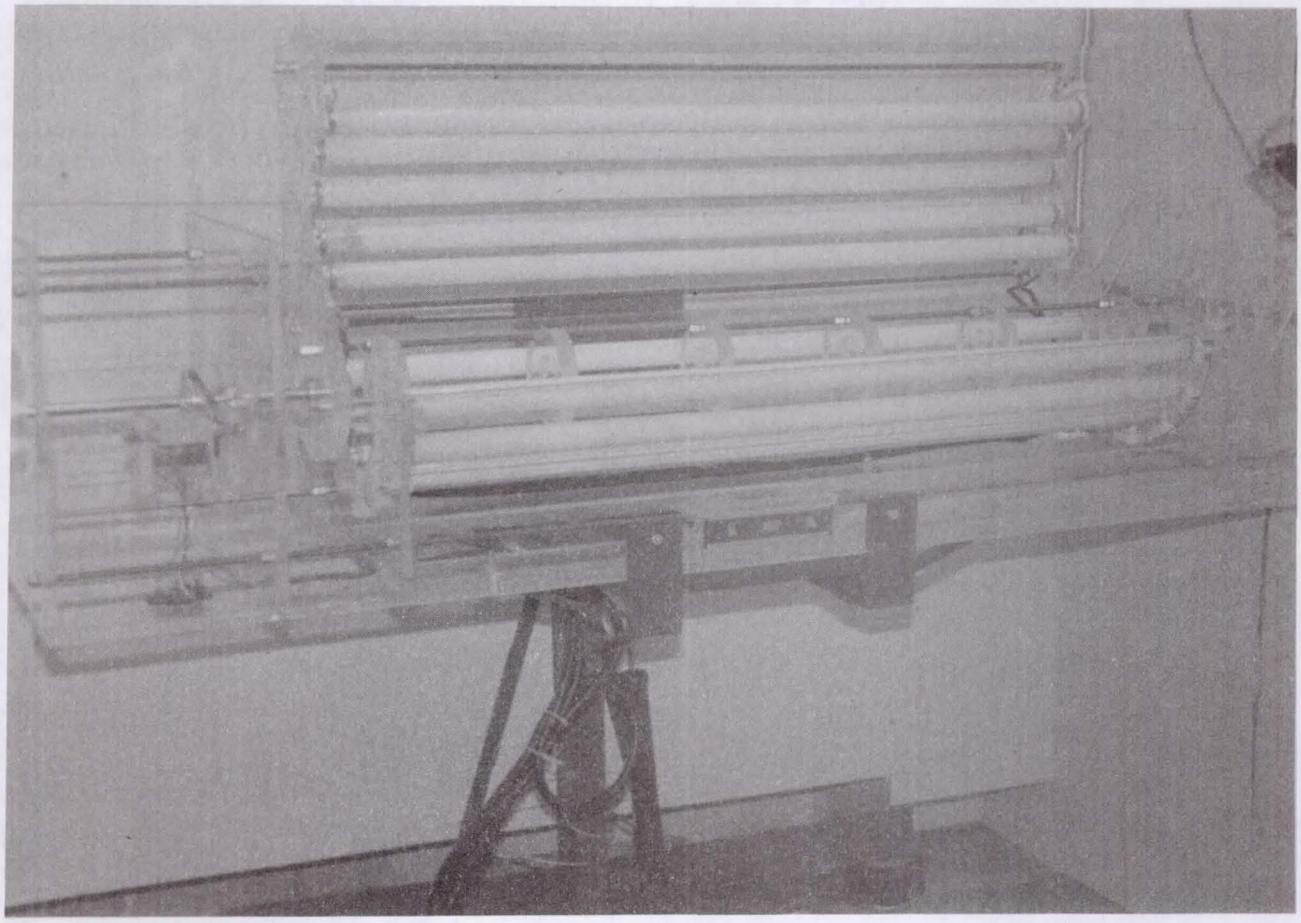

Figure 2. Clinostat design for hydroponically grown plants. Clinostat shown in the open position.

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ANALYSIS OF COMPONENTS OF THE GRAVITROPIC RESPONSE IN PLANTS USING PURIFIED PLASMA MEMBRANE VESICLES

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Description of Research

The Committee on Space Biology and Medicine (National Research Council) and the Workshop on Plant Gravitational and Space Research (NASA) both have emphasized the need for subcellular investigations into the mode of action and regulation of the transport of the plant growth regulator auxin and of calcium. An understanding of these processes should help elucidate the mechanism of signal transduction during the gravitropic response of plants.

Gravicurvature of plant stems is thought to be the result of differential growth in response to lateral transport of auxin (IAA). Recent studies suggest that Ca^{+2} redistribution is also necessary for gravicurvature. What is not yet clear is whether the Ca^{+2} movement is a cause or the effect of IAA movement, or whether the redistributions are independent. This question is difficult to answer *in vivo* due to the number of different cell types and intra- and extracellular compartments within which Ca^{+2} and IAA can be sequestered. To get around this problem, our research utilizes highly purified plasma membrane (PM) vesicles where polar auxin transport and Ca^{+2} fluxes can be studied directly.

Several essential steps involved in the gravitropic response are associated with the PM. It has been suggested that the gravitropic response may be regulated at the level of IAA and Ca^{+2} carriers located at the PM, either via the Ca^{+2}-binding protein, calmodulin, and/or via phosphorylation of the carriers by a Ca^{+2}-stimulated protein kinase. In addition, nothing is known in plants about PM Ca^{+2} transport mechanisms, since earlier studies have been carried out with microsomal rather than purified PM preparations. With those problems in mind, we are proceeding with the following investigations:

(1) Establishment of a system for *in vitro* studies on membrane transport components.
(2) Analysis of the interactions between Ca^{2+} and the transport of IAA.
(3) Examination of the role of protein kinases in modifying transport activity.
(4) Definition of the Ca^{2+} transport mechanisms in dicot shoot plasma membranes.

Accomplishments

As mentioned above, an essential component of this study is the use of purified plasma membranes for the transport studies. In the *1987-88 NASA Space/Gravitational Biology Accomplishments,* we reported that PM vesicles of extremely high purity (at least 95%) can be prepared from zucchini (*Cucurbita pepo*) hypocotyls which contain all of the necessary components of the polar auxin transport system thought to be involved in gravitropism. In this past year we have used this PM membrane system to:

(1) identify two polypeptides of 40 and 42 kDa which are labeled with high specific activity by ^{3}H-azido-IAA, a photoaffinity label, with characteristics very similar to the IAA

uptake carrier. These polypeptides were found not to label in the auxin non-responsive mutant of tomato, *diageotropica,* which is also altered in its gravity response.

(2) demonstrate that the published method for measuring Ca^{+2} uptake into plant membrane vesicles using chlorotetracycline is actually invalid and based on a biophysical artifact.

(3) identify and purify several PM protein kinases which are dependent upon Ca^{+2} for autophosphorylation activity.

Significance of the Accomplishments

Unfortunately, after much careful investigation, we have shown that the published method for measuring Ca^{+2} fluxes in membrane vesicles using chlorotetracycline was based on an artifact. We are currently investigating another method utilizing another Ca^{+2} probe, murexide, which appears much more promising. The identification of specific IAA binding proteins in the PM which are missing or altered in a gravitropic mutant presents the exciting possibility of demonstrating the role of IAA transport proteins in the gravitropic process. The identification and purification of Ca^{+2}-stimulated protein kinases also will allow us to assess *in vitro* the role of phosphorylation in modifying the activity of the transport components. We now plan to purify the IAA binding proteins and Ca^{+2}-stimulated protein kinases and generate specific antibody probes for them, define the basic mechanisms of PM Ca^{+2} fluxes and the effect of IAA on Ca^{+2} transport and, finally, ascertain at which step in gravitropism, perception, signal transduction, or response, each component is involved.

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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Description of Research

Plant development can be significantly impacted by physical disturbance in the form of wind, precipitation, shaking, vibration, and contact rubbing of plant parts. The usual plant response is inhibition of vegetative and reproductive development, but there are conditions under which mechanical disturbance has stimulated plant growth! Plants grown under otherwise low stress conditions (e.g., adequate water status, temperature, etc.) are particularly responsive to mechanical stress when grown in darkness or under low light levels. High irradiance lighting and/or a history of previous mechanical stress experience "harden off" plants to subsequent stress episodes and raise the threshold level of response. Non-uniform application of stress may cause tropistic growth responses, and plants hardened off by mechanical stress respond sluggishly to the usual tropistic stimuli (gravi-, photo-, etc.).

Increasing knowledge of mechanical stress/environment interactions as well as growing awareness that spacecraft in low Earth orbit have a physically "noisy" environment has raised concern among space biologists that the shuttle orbiter and proposed space station environment may not provide an adequate control for "g." Accelerometers placed onboard Spacelab have measured random "g-jitter" on the order of milli-g's (not μg) due to low-frequency vibration resulting from astronaut activity and machine operations.

Description of Research

The long-range goals of the research program are threefold: (1) to determine the physiological basis for plant response to mechanical stress; (2) to characterize the range of growth and developmental responses to mechanical stress; and (3) to extend the model to real spaceflight conditions to determine the threshold level of vibration within the range of frequencies encountered in orbiting spacecraft that modify growth and graviresponses.

The first goal is part of a continuing effort to identify the underlying basis for changes in growth rate caused by periodic seismic (shaking) or thigmic (contact rubbing) treatments. Present effort emphasizes work with sensitive dark-grown legume seedlings. Laura Coe, a graduate student, has measured real-time growth rate changes (microns min^{-1}) by 'Alaska' pea in response to either type of mechanical stress. She is also determining the relationship between stress ethylene, polar auxin transport, and the kinetics of growth rate changes. Russell Jones, a postdoctoral research associate, has been establishing a role for calcium in mediating inhibition of straight growth in soybean, and presently is characterizing effects of mechanical stress on cell wall extensibility.

With regard to the second goal, experiments have been conducted during the past year by R. Jones to determine the effectiveness of seismic stress on reproductive development of soybean when applied at different stages of vegetative and reproductive development. Jones and Coe together have quantified the irradiance dependence of stress sensitivity.

The Principal Investigator has been working directly with engineers and scientists at NASA Ames Research Center in connection with the third goal. A ground-based plant vibration study is planned to be done at Ames. Reorganization of a plant vibration flight experiment originally proposed by the PI in 1978 is anticipated as a result of these activities.

Accomplishments

(1) Monitoring elongation of dark-grown pea seedlings with an angular position-sensing transducer indicates ***an abrupt drop in growth rate in response to a single, brief episode of vibration or rubbing that occurs from seconds to minutes following the stress***. As much as an 80% inhibition of growth rate has been found to occur within 4 minutes of thigmo-stressing 20 times, followed by a more gradual recovery. Thirty-two minutes following treatment, thigmo-stressed plants had recovered to 70% of baseline growth rate, whereas plants that had been vibrated for 3 minutes already were growing 7% faster than controls. ***A net 15% stimulation of epicotyl elongation 24 hr after a vibration episode is not uncommon***, whereas thigmic stress almost always results in significant growth inhibition.

(2) L. Coe also found that a single episode of ***thigmic stress inhibited basipetal auxin transport by pea epicotyls to the same extent that it inhibited epicotyl growth over a 24-hr period (40-50%)***. Further characterization of this response is ongoing, including effects of vibration on polar auxin transport.

(3) Previous work in our laboratory has shown that stems of plants having been seismic-stressed for some time become fibrous and flexible, and can be reversibly bent into unnatural shapes. On the other hand, the stems of undisturbed control plants are stiff and brittle. They can be easily snapped if displaced slightly from their equilibrium position. Russell Jones has extended this approach to measuring shorter-term changes in cell wall extensibility compliance that occur in the hypocotyls of dark-grown soybean seedlings during a 24-hr period following application of a single thigmic stress to the apical hook. The cell enlargement zone was then excised and boiled in methanol to remove the influence of the protoplast. An extensometer measured the extensibility of hypocotyl sections in the axial direction, and ***mechanical stress was found to increase wall extensibility as much as 54% relative to that of unstressed controls. The elastic component of total extensibility was most affected by stress.*** Methanol-boiled hypocotyl sections were more extensible at pH 5.4 than pH 7.4. Pronase did not appreciably affect wall extensibility compliance at either pH.

Significance of the Accomplishments

Many early-generation plant flight experiments will be conducted either in darkness or under the low-irradiance lighting capabilities of present and projected plant growth units. The considerable sensitivity and rapid response of such plants to mechanical disturbance suggests that for short-term experiments seeds rather than seedlings should be sent up to avoid the teeth-jarring vibration that occurs during launch until main engine cutoff. Once on orbit, the same reasons beg the question of whether or how much vibration isolation is required to assure the success of future gravimorphism and gravitropism studies to be conducted in low Earth orbit.

The finding that elastic extensibility of cell walls is enhanced by mechanical stress has opened new questions regarding what biophysical aspect of cell wall enlargement becomes most limiting as a result of an effective mechanical stress experience. It does not appear

that the primary wall becomes irreversibly rigid or stiff. Much additional work remains to be done in this area.

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HOW GRAVITY AFFECTS PLANT GROWTH AND DEVELOPMENT

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Description of Research

The purpose of this research is to determine how gravity affects plant growth and development. We have taken several approaches to studying this problem. We have: (1) studied how calcium affects growth and gravitropism by quantifying and localizing endogenous calcium in graviresponding roots; (2) studied how roots respond to gravity by determining when and where gradients of gravitropic effectors form in graviresponding roots; (3) characterized the pathways by which gravitropic effectors could move from the root cap to the root in *Zea mays;* (4) studied the influence of other ions in gravicurvature by studying how asymmetries of ions such as aluminum influence root growth; and (5) examined plants flown aboard flight 61-C of the space shuttle *Columbia.* We flew seeds and seedlings of corn, onion, and mustard spinach, and examined their ultrastructures both qualitatively and quantitatively.

Accomplishments

(1) We found similar microgravity-induced changes in all of the flight tissues examined to date (our studies have concentrated on corn, but our preliminary examinations of onion and spinach are showing the same trends). ***Microgravity significantly affects the structure and function of plant cells.***

(2) Microgravity-induced changes in ultrastructure are cell-specific and organelle-specific: microgravity tends to decrease the relative volume of plastids, mitochondria, and dictyosomes, and tends to increase the relative volume of lipid bodies, hyaloplasm, and the vacuome.

(3) A gradient of gravitropic effectors occurs in the mucigel surrounding horizontally oriented roots. Also, primary roots of *Zea mays* cv. Ageotropic are agravitropic and produce no mucilage. Adding mucilage or mucilage-like material to the root tip induces graviresponsiveness. Washing off the mucilage again renders the root nonresponsive to gravity. These results strongly suggest that ***gravitropic effectors can move apoplastically, possibly in mucilage.***

(4) Removing the epidermis and cortex from one side of a vertically oriented root induces curvature toward that side of the root, even if the tip of the root is submerged in water. Applying mucilage or mucilage-like materials to the root eliminates or dramatically diminishes this response. These results support the suggestion that gravitropic effectors may move apoplastically.

(5) Cells of seedlings grown on a rotating clinostat have significantly different ultrastructures than those grown in microgravity. These results indicate that ***clinostating does not mimic the ultrastructural effects of microgravity in plant cells.***

Significance of the Accomplishments

Our results indicate that gravity exerts specific effects on the structure and function of plant cells. These effects are similar throughout a plant and in different types of cells. For example, plants grown in microgravity contain much less starch than those grown on Earth. If our conclusions hold true for long-term studies, then the caloric value of starch-rich plants grown in space will probably be less than that for plants grown on Earth. Also, our results indicate that clinostating does not mimic the ultrastructural effects of microgravity in plant cells.

Our results indicate that the apoplast may be an important part of the transport pathway for movement of gravitropic effectors between the root cap and root. This information should help us identify the signal and understand how it functions.

Publications

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GRAVITROPISM IN *ARABIDOPSIS THALIANA:* A GENETIC APPROACH

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Description of Research

Our ultimate objective is to achieve an understanding at the molecular level of the mechanism whereby a plant measures and responds to a gravitational stimulus. Toward that end, this project is developing a collection of mutant strains of *Arabidopsis* with alterations in gravitropism. These lines will permit the identification of the elements in the transduction pathway, and will permit an analysis of the interrelations between the two sensory responses, gravitropism and photropism.

The work is divided into several different aspects. First, a family of mutants has been identified with alterations in gravitropism and phototropism. Second, individual mutants are characterized physiologically and genetically. Third, particularly interesting mutants will be mapped with respect to morphological markers and restriction fragment length polymorphisms in preparation for cloning the genes of interest. This work is related to the goals of the NASA Space Biology Program to identify the components in the gravity-sensing system of plants, and to determine the mechanisms whereby gravity interacts with other environmental factors to control the physiology and morphology of plants.

Accomplishments

(1) Based on 59 mutants with alterations in gravitropism, we find that gravitropism by the root and by the hypocotyl may be independently altered. Thus, ***it appears that the pathways for root and hypocotyl gravitropism share some elements, but have other elements which are organ specific.***

(2) Based on mutants with alterations in gravitropism and phototropism, we find that these pathways may be altered simultaneously or separately. Thus, the pathways for phototropism and gravitropism share at least one common element.

(3) Part of the heterogeneity in hypocotyl gravitropism by a population of plants results from the dependence of curvature on the position of the hook with respect to the gravitational vector. It is proposed that the position of the hook with attached cotyledons affects curvature and not stimulus perception.

Significance of the Accomplishments

The utility of this genetic approach is demonstrated by the conclusion that phototropism and gravitropism share at least one common element, and by the conclusion that root and hypocotyl gravitropism differ by at least one element.

Publications

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CALCIUM MESSENGER SYSTEM IN GRAVITROPIC RESPONSE IN PLANTS

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Description of Research

Investigations during the last decade indicate that calcium is involved in gravity signal transduction in plants. However, the exact molecular mechanism(s) related to calcium action is not clearly understood. It is believed that protein phosphorylation plays an important regulatory role in transducing and amplifying signals. In our laboratory we have been investigating calcium-calmodulin-regulated protein phosphorylation and gene expression in root tips and their role in gravitropic bending.

Accomplishments

We have observed rapid in vivo calcium- and calmodulin- dependent changes in protein phosphorylation in corn roots. These changes are localized primarily in the root tip, the site of gravity signal perception. Many of the effects of calcium ions in plants are mediated by the calcium-binding regulatory protein, calmodulin. We have recently cloned and sequenced a plant calmodulin cDNA. We are currently investigating the expression of the calmodulin gene and its role in signal transduction. ***Our recent work has revealed changes in calmodulin gene expression in root tips.*** Whether and how the changes in the expression of the calmodulin gene are involved in signal transduction is being investigated. ***Furthermore, we have isolated a calmodulin-dependent protein kinase from corn, and we are further purifying it to investigate its possible role in gravity signal transduction.***

Significance of the Accomplishments

Based on the existing evidence, we propose the following model (Figure 1) describing the biochemical and molecular events involved in the transduction of the gravity signal.

Publications

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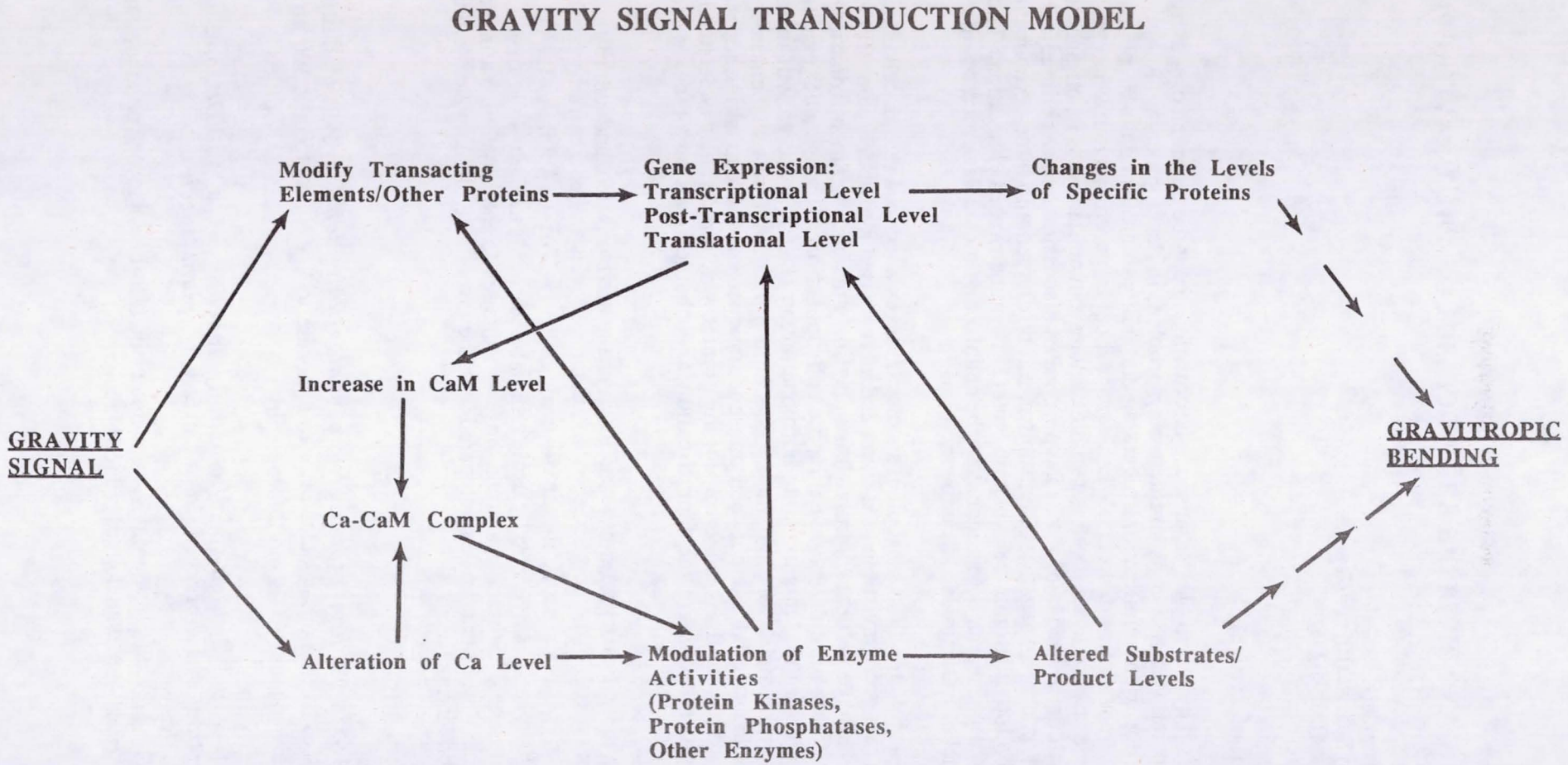

Figure 1. Diagram illustrating the proposed sequence of events involved in the transduction of the gravity signal. Ca = calcium; CaM = calmodulin

CELLULAR AND MOLECULAR PROCESSES OF THE GRAVITROPIC RESPONSE

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Description of Research

The mechanism by which plants transduce information about the direction of gravity into a predictable pattern of growth is an interesting problem in developmental biology and in addition has important ramifications regarding our ability to grow and utilize plants in the microgravity environment of space. When a plant shoot is placed in a horizontal position it begins to curve upward in a smooth arc within minutes and reestablishes its original vertical orientation within several hours. For the purpose of discussion and experimentation this phenomenon, known as negative gravitropism, can be divided into three components: (1) gravity perception, (2) signal transduction, and (3) asymmetric cell elongation. The long-range goal of my research is to understand the cellular and molecular processes that occur between signal transduction and asymmetric growth.

Asymmetric shoot growth in most plant species involves a reduced rate of cell elongation on the upper side of a gravistimulated shoot and an increased rate of cell elongation on the lower side relative to vertical controls. It is quite likely that an asymmetric distribution of some growth factor must precede asymmetric cell elongation. There is a large volume of literature suggesting that in shoots the hormone, auxin (IAA), is the growth factor that modulates asymmetric shoot elongation. This in turn makes it likely that auxin-specific receptors are an essential link between transduction events and the resulting response of gravistimulated tissue. Thus, a logical starting point toward a molecular understanding of the gravitropic response is the identification and characterization of auxin-specific receptors.

The approach that we (Hicks, Lomax, and Rayle) have used to isolate and identify putative auxin receptors involves the preparation of membrane vesicles and photoaffinity labeling of membrane polypeptides using the auxin analogue, ^{3}H-$5N_3$-IAA (azido-IAA). This technique has advantages over simply using radiolabeled auxins to study and isolate ligand-receptor complexes. Foremost among these advantages is the covalent nature of the photolyzed azido-receptor complex compared with the low affinity, noncovalent binding of non-derivitized auxins.

Accomplishments

(1) ***Auxin-binding proteins were photoaffinity labeled*** by the addition of ^{3}H-$5N_3$-IAA to membrane vesicles prior to exposure to UV light (15 sec; 300 nm) and detected by subsequent PAGE and fluorography.

(2) At -196°C, ***high-specific-activity labeling of a 40-kDa and a 42-kDa polypeptide was observed in a variety of plant species.***

(3) The labeled polypeptides were determined to be of low abundance and the addition of IAA and auxin analogues reduced labeling.

(4) Shoots of the tomato mutant *diageotropica (dgt)* were found to have reduced amounts of the 42 kDa labeled polypeptides relative to wild-type controls.

(5) The above findings and physiological studies of *dgt* and wild-type tomato seedlings suggest the radiolabeled polypeptides are auxin receptors.

Significance of the Accomplishments

We have shown that the azido-labeled 40-42 kDa membrane proteins are (a) ubiquitous in plant tissues that respond to IAA; (b) of low abundance; (c) saturable with increasing concentrations of IAA; and (d) capable of binding specific analogues that are also active auxins or specific antagonists. These data suggest that the two polypeptides are part of a physiologically important auxin receptor system. It is hoped that further characterization of these polypeptides will make it possible to dissect the mechanism of auxin action and thus provide us with a clearer understanding of plant gravitropism.

Publications

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REGULATION OF WALL ENZYMES DURING LIGHT-STIMULATED GRAVITROPISM

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Description of Research

Light greatly accelerates the gravitropic response of roots, coleoptiles, and stems in a wide variety of plants. This indicates that some cellular response initiated by light affects at least one of the gravity-induced cellular responses necessary for gravitropism. Our research objective is to identify one or more of these responses, and thus clarify the cellular mechanisms regulating gravitropic growth.

The best characterized photoreceptor for light-regulated gravitropism is the pigment phytochrome. The photoconversion of this pigment to an activated form, called Pfr, has rapid and dramatic effects on the straight growth of coleoptiles and stems, and accelerates the gravitropic curvature of coleoptiles, stems, and roots of various plants. Understanding the mechanisms by which Pfr regulates growth should enlighten how light alters gravitropic growth. Recent research has supported the hypothesis that phytochrome modulates growth primarily by altering wall extensibility, and that wall extensibility is partially controlled in many plants by wall-localized enzymes whose activity affects the cross-linking of wall structural elements.

Peroxidases are prominent among the wall enzymes proposed to alter wall extensibility, because they are known to promote the cross-linking of wall macromolecules. Furthermore, there is an inverse relationship between the activity of wall peroxidases and the growth of cell walls: those hormonal and environmental stimuli that inhibit growth stimulate wall peroxidase activity, and those stimuli that inhibit growth stimulate wall peroxidase activity. Further interest in peroxidase involvement in growth derives from the fact that Ca^{2+} ions, which decrease wall extensibility, stimulate both the activity and the secretion of wall peroxidases, so wall peroxidases could help account for the inhibition of growth by Ca^{2+}. The asymmetric accumulation of Ca^{2+} in the walls of cells along the slower growing (upper) side of a horizontally positioned coleoptile is thought to contribute to the reduced growth of these cells during gravitropic (upwardly curved) growth. Thus, tests of the interrelationships of calcium and wall peroxidase activity and of wall peroxidase and growth would be very relevant to hypotheses proposing a role for Ca^{2+} in mediating gravitropic growth.

Last year we reported that the promotion of growth of corn coleoptiles by red light was accompanied by a decrease in the extractable level of a 98 kDa acidic wall peroxidase, as assayed by an immunoassay using a monoclonal antibody (mWP3) specific to that peroxidase. This year we report that the red light-induced inhibition of growth of corn seedling mesocotyls is accompanied by an increase in the extractable level of the 98 kDa peroxidase and that the peroxidase changes in both the coleoptile and mesocotyl precede any detectable growth change.

Accomplishments

(1) An enzyme-linked immunoadsorbent assay (ELISA) using mWP3 reveals that ***within 30 min following the photoactivation of phytochrome in corn mesocotyls, the quantity of immunodetectable peroxidase that is extracted from mesocotyls increases by more than 65%.*** This change is opposite to that induced by red light in coleoptiles (see above), which also show an opposite growth response to red light.

(2) We developed a video microscopy procedure for recording rapid corn growth responses to red light. This procedure uses a video camera sensitive to infrared (IR) light to record the growth of plant tissue magnified through a stereo dissecting microscope, using as the only illumination source an IR light system that does not affect phytochrome-regulated growth responses. With this system we were able to record changes in growth rate as small as 0.07 mm/hr and to show that R-induced growth changes in coleoptile segments have a lag time of about 20 min, significantly longer than the lag time for the R-induced wall peroxidase change (= 5-10 min).

(3) Using the video microscopy procedure and an equally sensitive extensiometer procedure, we documented that ***the R-induced growth changes in corn mesocotyls had a lag time of about 35 min, about 5 min longer than that for R-induced wall peroxidase changes in these organs.***

(4) ***We have purified a 60 kDa cellulase from the cell walls of corn seedlings to greater than 80% purity*** in quantities sufficient for biochemical analysis.

Significance of the Accomplishments

Finding #1: The close correspondence of opposite wall peroxidase changes and opposite growth changes induced by red light in coleoptiles and mesocotyls of the same corn seedlings indicates that the 98 kDa wall peroxidase could be an important participant in transducing the light signal into growth changes.

Finding #2: White or red light is known to alter the gravitropic responses of the shoots and roots of many plants. The method of sensitively recording small R-induced growth changes using non-activating IR light to "illuminate" the responding plants will permit similar recordings of gravitropic responses without the interference of actinic light.

Findings #2 and #3: The fact that the R-induced peroxidase changes precede any detectable growth changes induced by R in both coleoptiles and mesocotyls allows for the possibility that the 98 kDa wall peroxidase could play a causal role in regulating the growth responses of these organs to light. The methods used for these experiments can now be applied to determine whether changes in the immunodetectable level of the 98 kDa wall peroxidase also correspond with growth changes that occur along the upper (slower growing) and lower (faster growing) sides of upwardly curving coleoptiles and mesocotyls during gravitropic growth.

Finding #4: Purification and biochemical analysis of wall enzymes such as peroxidases and cellulases will be a requirement for understanding both their export to the wall and their turnover and regulation in the wall. This knowledge, in turn, will be prerequisite for understanding enzyme involvement in wall extensibility changes, such as those that are critical for gravitropic growth.

Publications

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CELLULAR POLARITY AND INTERACTIONS IN PLANT GRAVIPERCEPTION

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Description of Research

Our long-term goal is to understand the mechanism(s) of gravity perception in higher and lower plants. Although it had long been thought that the movement of amyloplasts — heavy, starch-filled plastids — probably triggers perception, the recent isolation of several starch-deficient, but gravity-perceiving, mutants indicated that amyloplasts are not necessary for perception. However, last year we demonstrated that starchless roots of *Arabidopsis* are less sensitive to gravity than are wild-type roots with amyloplasts, and we therefore concluded that starch (or presumably the weight it confers) is necessary for full sensitivity. Furthermore, since the starchless plastids are relatively dense and are the most moveable component in mutant root cells, we suggested that the starchless plastids function in perception in mutant roots.

Recently we have started to characterize a starch-deficient mutant of *Nicotiana sylvestris* with respect to its starch content and its sensitivity to gravity. Additionally, we are analyzing the kinetics and cell biology of gravitropism in the moss *Ceratodon* which has filaments of cells (protonema) which grow upward in the dark.

Accomplishments

(1) ***A tobacco mutant with reduced starch has very disoriented stems (hypocotyls). Mutant roots are oriented, but are less graviresponsive than WT roots.*** Although initially described as starchless by its developers, electron microscopy and iodine potassium iodide staining reveals that plastids in the stem endodermis (starch sheath) and in the central cells of the rootcap do have small amounts of starch. Mutant roots grow down but with less sensitivity to gravity than wild-type roots. Mutant stems (grown in the dark) are severely disoriented with respect to gravity whereas wild-type stems grow straight up (Figure 1).

(2) ***When protonema of the moss Ceratodon are reoriented 90^{o}, the cell outline changes within minutes, and the tip often grows the "wrong way" before ultimately curving upwards.*** Upward curvature starts as early as 30 min after reorientation, but even 5 min after reorientation, the upper flank of the cell tip changes form. The time course of events is observed using infrared videomicroscopy and a horizontally mounted microscope.

(3) Tip cells of *Ceratodon* protonema have a zonation not previously described. Amyloplasts exist in distinct zones, but sedimentation only occurs behind the region of growth. The conclusion that this zone is specialized for lateral but not lengthwise amyloplast movement in the cell is confirmed by low g centrifugation studies.

GRAVITROPIC RESPONSE OF MUTANT AND WILD TYPE TOBACCO SEEDLINGS

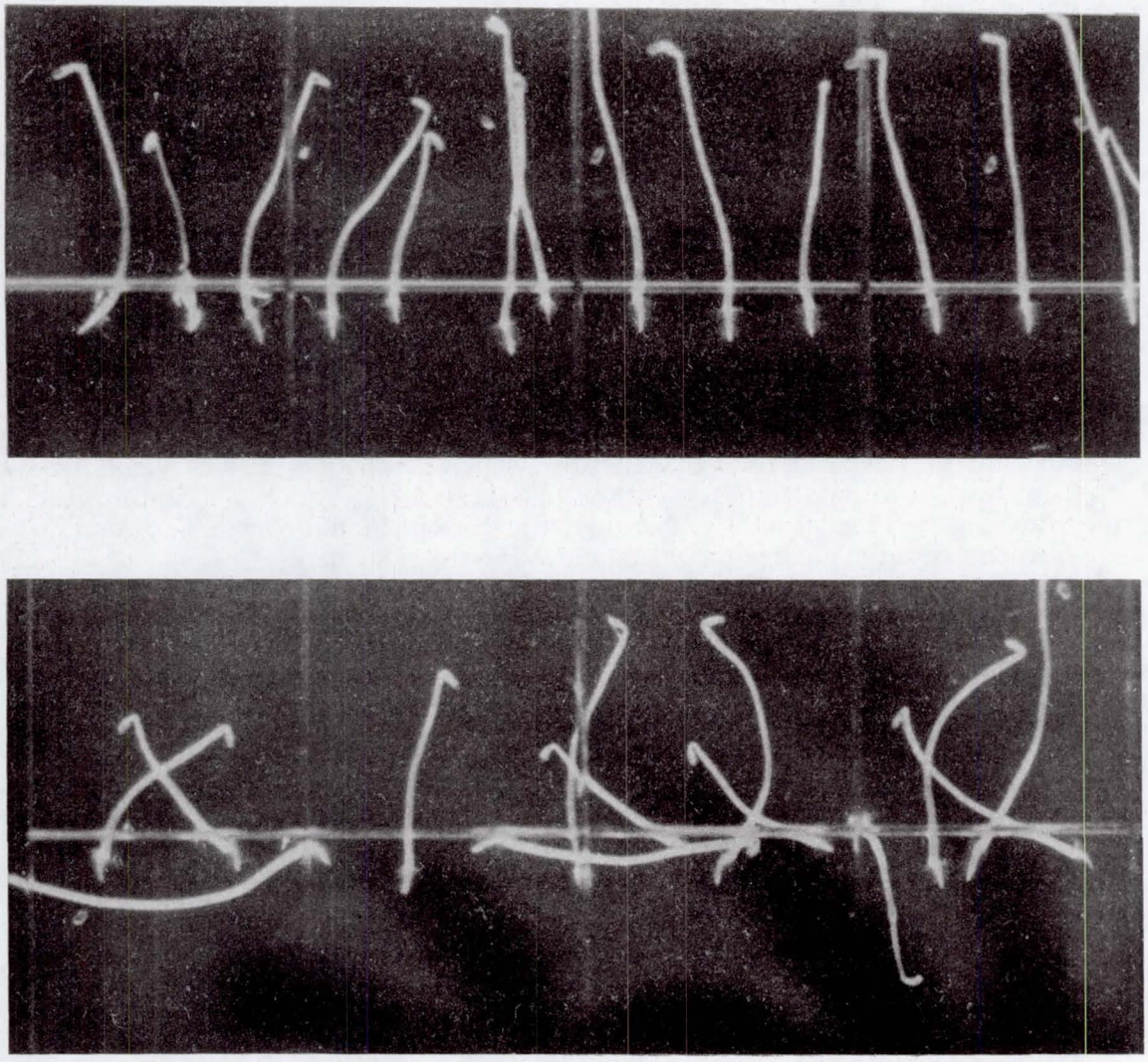

Figure 1. *Nicotiana* seedlings grown in the dark on nutrient supplemented agar. The gravity vector is towards the bottom of the figure. Wild-type seedlings (top) have a full complement of starch and the stems (hypocotyls) are more or less vertically oriented. Mutant (bottom) stems have much less starch and are severely disoriented. Distance between vertical reference lines (grid pattern on petri dish) is 1.3 cm.

(4) ***Amyloplast sedimentation in protonema occurs before upward curvature begins.*** Significant early sedimentation was observed in populations and individual protonema viewed in time-lapse studies.

(5) ***Reorientation of the protonema results in an increase in microtubule density close to the part of the cell on the lower flank where curvature develops*** (Figure 2). Immunofluorescence also reveals that microtubules are oriented predominantly along the long axis of the cell throughout the protoplast and that microtubules are closely associated with amyloplasts. The higher concentration of microtubules near the lower wall was found in the majority of, but not in all, protonema reoriented for varying periods. A higher concentration was never found near the upper wall.

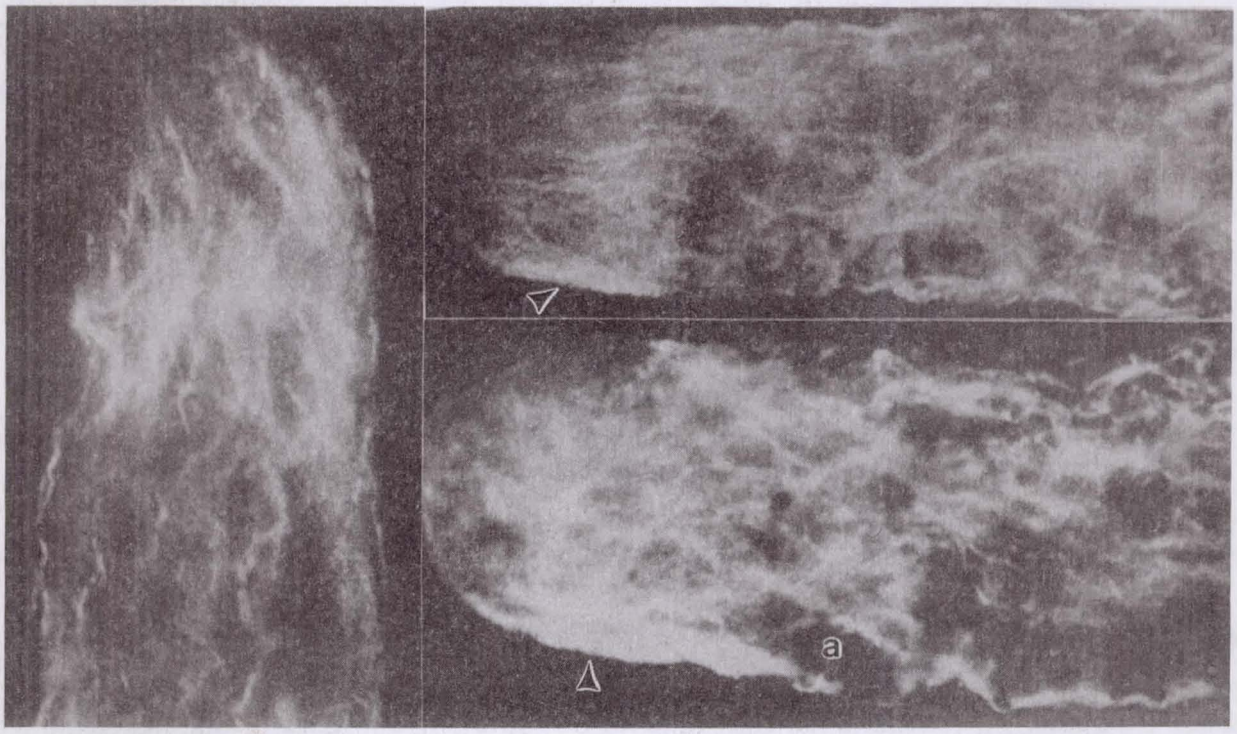

Figure 2. Microtubules (immunofluorescence) in the tips of protonema of the moss *Ceratodon*. Gravity is towards bottom of all micrographs. Microtubules in vertical protonema (left micrograph with tip at top) are present in roughly equal numbers on both sides of the cell. In horizontal protonema (right micrographs with tip at left), microtubules are present in a higher concentration near the bottom flank (arrowheads), compared to the top. Amyloplasts (a), which are distinguishable as dark (non-fluorescent) regions, are sedimented near the lower wall just behind the region where microtubules become concentrated and behind the region where the lower wall grows faster than the upper wall.

Significance of the Accomplishments

Finding #1: *Arabidopsis* mutant TC7 is starchless in the roots and is approximately 8-20% as sensitive to gravity as the wild-type. The lowered sensitivity of the *Nicotiana* mutant demonstrates in another genus that starch is necessary for full gravitropic sensitivity. Since the *Nicotiana* mutant does have some starch in its plastids and its roots appear to be closer in sensitivity to the WT than TC7, this may suggest that the hypothesized relationship between plastid buoyant weight and sensitivity is probably not linear. Additionally, we hypothesize that a small amount of starch is more effective in orienting roots than stems.

Findings #2-4: This is the first in-depth description of a plant system whose growth is oriented with respect to gravity in which the same cell perceives and responds to gravity and in which amyloplasts appear to act as statoliths (trigger perception). Wrong-way curvature has often been described in higher plant roots and shoots, and it is intriguing that it is also found in a much more primitive plant. The initial changes in cell outline represent one of the faster responses to gravity known for the plant kingdom and they indicate that tip growth in these cells is tightly controlled by gravity.

Finding #5: These observations demonstrate the potential usefulness of this system for studying the cell biology of gravity perception and differential growth. To our knowledge, this is the first report of an effect of gravity on the organization of the cytoskeleton in plants. Future research will help to determine whether amyloplast sedimentation directly or indirectly produces the higher concentration of (movement of?) microtubules and whether microtubule concentration is proportional to the extent of cell and cell wall growth in localized regions.

Publications

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GRAVITROPISM IN DICOT STEMS

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Description of Research

During recent years, we have concentrated on the role of auxin (indole acetic acid, IAA) in the gravitropic response of stems. While most research in this field assumes that gravitropic stem bending is controlled by a gradient in auxin concentration within the stem, we have emphasized an alternative mechanism in which the response of the tissue to the auxin changes, rather than the concentration of auxin changing. We have presented evidence that, when a stem is turned to the horizontal, cells in the bottom half increase their sensitivity to auxin relative to cells in the top half. We obtain dose-response curves to auxin solutions over a wide range of concentrations (zero, 10^{-8} to 10^{-2} M IAA). The maximum growth response of top or bottom surfaces (at their optimum auxin concentrations) indicates V_{max} sensitivity, and the concentration required to produce half of V_{max} shows K_m sensitivity. Because lower tissues apparently have a near saturating amount of auxin, a true K_m sensitivity cannot be determined, but the concentration that produces maximum growth is also an indication of K_m sensitivity. Both V_{max} and K_m sensitivities are greater in lower tissue than in upper tissue; the difference in K_m sensitivity may approach two or three orders of magnitude.

Publications of our results and presentations at meetings during the past year elicited questions about our methods, and there is a considerable variability from experiment to experiment and even among the replications within a given treatment. These circumstances, along with a move to a new laboratory that necessitated setting up our equipment again, suggested that it was time to take a fresh look at some basic questions of technique.

Accomplishments

(1) It was suggested (at a meeting) that our results might depend upon the presence of potassium ions and/or the osmotic potentials of our solutions. We performed several experiments with K^+ and with mannitol and found no responses except at unrealistically high concentrations. Another suggestion was that our results might be influenced by tensions in the water columns (xylem) of the segments and that this could be overcome by cutting the segments under water. We tried this but could see no difference from plants that were not cut under water. It also seemed possible that the white oil paint used to mark segments might have influenced our results, but again tests were negative.

(2) In previous experiments with mature stems instead of seedling hypocotyls, we had observed that physiological differences caused by gravistimulation and bending were greatly accentuated if the stems were restrained in a horizontal position so they could not bend up. When they were released from restraint, bending occurred suddenly (within 1 to 10 sec). We thought we could apply this principle to our dose-response experiments, so we expended considerable time and effort in attempting to do so. Unfortunately, none of our trials were successful. We finally abandoned the attempt.

(3) Marking our hypocotyl sections with white, titanium-oxide oil paint is tedious, and we frequently found it difficult to get the marks in just the right places. Thus we now use shorter unmarked hypocotyl segments, all cut to a standard length. We have constructed some simple equipment to accomplish this. Next, we modified our equipment to use at least 20 hypocotyl segments (instead of 10) in each auxin concentration. Although this does not eliminate the variability, it does improve the statistics somewhat. We have also doubled the number of auxin concentrations to obtain more points on our bending and growth curves. These two steps mean four times as many segments to measure with our digitizer/computer system. To facilitate the increased number of segments, we developed an approach in which three points are measured on the bending segment, and these three points are used to calculate the curvature and the distance along the surface (between the two most distant points). A fourth point on the opposite side of the segment gives distance along that surface as well (because the segments have a uniform thickness). Two more points at calibration lines on the equipment allow the data to be expressed as actual lengths. We developed a program in BASIC that uses the data produced by the digitizer to make these calculations. Of course, this technique assumes that each hypocotyl section is a segment of a circle, and sometimes this is not true. We are checking the technique against more elaborate methods that we know are reliable.

(4) Our new laboratory has not yet had temperature control installed in the experiment rooms. Our experiments in the old laboratory were done at 32°C, but those done in the new lab could only be done at room temperature (18 to 22°C). The results were quite different in one way: ***The downward bending at high auxin concentrations was considerably less pronounced and occurred only at higher concentrations than had produced the effect in the old lab.*** We are anxious to determine if the temperature difference is the key to these different results.

(5) The gravitropic memory experiments of Brauner and Hager, first published in 1958, clearly separate the perception of the gravity stimulus from the plant response. Sunflower seedlings were decapitated and left for four days during which time they became somewhat depleted in auxin and much less sensitive to auxin. When they were gravistimulated in this condition, they failed to respond, but if they were returned to the vertical and supplied with auxin, bending occurred in the expected direction. This technique opens a number of avenues for study of the gravitropic process, and it is especially well suited to studies of tissue sensitivity to auxin. Our initial results (with labelled IAA) showed that there was no auxin gradient in the stem. Because bending is completely dependent upon the added auxin, it must depend upon something other than auxin concentration, and tissue sensitivity to auxin is the prime candidate. We spent part of last year adapting the memory experiments to our system in which stem segments are completely immersed. (In the original experiments, auxin was added in small glass tubes attached to the cut end of the stem.) Although there is still room for improvements, ***we are able to duplicate the Brauner/Hager experiments.***

Significance of the Accomplishments

Finding #1: Because our finding that sensitivity plays a significant role in gravitropism of stems is contrary to the venerable auxin-transport model, our results have been met with some skepticism. Thus, it is important to examine any possible sources of error. We have eliminated K^+, osmotic potentials, water relations, and oil paint as such sources of error.

Finding #2: It was a disappointment to find that the restraining technique will not work with tender hypocotyl segments, but we can lay the issue to rest.

Finding #3: The new techniques we are developing should improve the quality of our dose-response curves and thus make them more convincing to other workers.

Finding #4: The possible importance of temperature in the control of tissue sensitivity to auxin was an unexpected finding and could be significant. We hope to confirm or reject it in the not-too-distant future.

Finding #5: The gravitropic memory experiments can provide strong evidence for a sensitivity model, as indicated above. At least one other worker in the field was unable to repeat them, so we are gratified that we can do so.

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AMINE OXIDASE AND PEROXIDASE REGULATION OF DIFFERENTIAL GROWTH RESPONSES IN GRAVISTIMULATED PLANTS

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Description of Research

Our long-term goals are to understand how plants are influenced by gravity, with respect to their ability to adapt to and survive in the space environment. The biochemical and physiological processes by which plants regulate growth in response to altered gravity vectors or changes in acceleration within the gravitational field are poorly understood. An elucidation of such mechanisms will facilitate our understanding and prediction of plant growth responses in both normal Earth (1 g) and hypogravity environments that would be encountered in space.

Our specific goal is to determine the extent to which peroxidase-mediated cross-linking mechanisms, and the expected inhibition of cell wall extensibility and cell growth resulting from such cross-linking, contribute to short-term, largely reversible tropistic growth responses in plants (i.e., differential growth, or bending of a plant organ in response to changed orientation with respect to the gravity vector). More permanent, irreversible cross-linking of this type is thought to be responsible for lignification processes, which provide long-term structural support to plants, and there is evidence that these processes are also influenced by gravity.

Our research is designed to test a model in which (1) amine oxidases in the plant cell wall would generate H_2O_2 (hydrogen peroxide), as a result of polyamine substrate oxidation; (2) wall-localized peroxidases would utilize this H_2O_2 in carrying out cross-linking of phenolics in the wall, thus influencing wall rigidity and cell growth; and (3) differential growth accompanying tropistic curvature of the gravistimulated organ results from gradients in the activities of amine oxidases and/or peroxidases. Previously, we have used both cytochemical and biochemical methods to show that amine oxidase activities are the same across such organs, while their amine substrates, as well as wall-localized peroxidases, exhibit a lateral asymmetry that is consistent with growth responses predicted by the proposed model. In future experiments, we will examine the extent to which peroxidase activities can be modulated by exogenous applications of Ca^{2+} ion or auxin, two putative "transduction" signals that are thought to play a role in tropistic growth responses.

Accomplishments

(1) We have ***developed a sensitive HPLC method which permits routine analysis and quantitation of polyamines in plant tissues.***

(2) We have demonstrated that ***exogenous, asymmetrically applied H_2O_2 is capable of inducing differential growth responses in corn coleoptiles.***

(3) We have developed methods for studying changes in cell wall isoperoxidase profiles in response to gravistimulation or exogenously applied growth regulators.

Significance of the Accomplishments

Finding #1: Accurate quantitation of polyamine substrate levels in the cell wall was required in order to support the current model for growth regulation involving amine oxidases. The development of a sensitive HPLC assay method has permitted routine analyses of wall polyamine titers down to picomole levels. In addition, a wider range of amines can now be analyzed by a single chromatographic procedure.

Finding #2: A key aspect of the model is H_2O_2 involvement in initiating the differential growth response via peroxidase-mediated cross-linking reactions. Asymmetric application of H_2O_2 to either vertically or horizontally oriented corn coleoptiles induces bending toward that side, suggesting that H_2O_2-induced growth inhibition contributes to this response. The kinetics of this response and the extent to which this curvature is reversible are presently under investigation. H_2O_2 produces markedly decreased effects in coleoptiles pretreated with aminotriazole, a peroxidase inhibitor, but it is not yet clear whether this inhibitor affects short-term growth responses.

Finding #3: We have shown that there is a lateral asymmetry in ionically bound peroxidase activities across gravistimulated pea epicotyls and corn coleoptiles. We are interested in knowing whether specific isoperoxidase activities are induced upon gravistimulation and the extent to which these enzyme activities are regulated by Ca^{2+} ion and auxin levels in the cell wall. Several studies have identified isoperoxidases whose activity is modulated by one or both of these growth regulators. We want to explore the possibility that the lateral Ca^{2+} asymmetry that we previously demonstrated in coleoptiles (Slocum and Roux, *Planta* 157: 481, 1983) might influence growth by increasing the activities of certain wall peroxidases on the slower-growing side of the organ, where Ca^{2+} accumulates in the cell wall. We are attempting to characterize Ca^{2+}-dependent peroxidase activities from the walls of gravistimulated coleoptiles by non-denaturing PAGE and in gel staining methods, but have encountered difficulties in obtaining adequate enzyme yields from wall extracts. Presently, we are investigating different extraction methods to improve the efficiency of peroxidase extraction.

Publications

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ANIMAL PROJECTS

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EFFECT OF SKELETAL UNLOADING ON BONE FORMATION

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Description of Research

The long-range goal of our research program is to understand the effects of gravity on skeletal development and bone metabolism. Our present objectives are to define the effects of gravity or mechanical stress on bone formation and resorption, and to determine the mechanisms (hormonal or paracrine) by which mechanical stress is coupled to bone cell activity.

Bone is a dynamic, living tissue. It continually is undergoing change, or remodeling, which involves a delicate balance between bone formation and bone resorption. This balance is influenced by systemic hormones such as parathyroid hormone, glucocorticoid hormone, growth hormone, and the vitamin D metabolites, as well as local factors such as blood flow, neuromuscular activity, and mechanical stress. Recently, a number of cytokines have been observed to stimulate or inhibit bone formation and resorption. Some of these cytokines, such as insulin-like growth factor-1 (IGF-1), transforming growth factors, and fibroblast growth factors, have been identified in bone. IGF-1 production by bone appears to be regulated by growth hormone. Such cytokines may participate in coupling mechanical stress to bone cell activity, or the activity of one type of cell to activity of another type of cell, for example, osteoblast activity to osteoclast activity. Our research over the past year has been directed toward elucidation of the role that the systemic hormones play in coupling the mechanical stress of weight-bearing to the cellular response of bone formation as well as beginning the assessment of the role of locally produced cytokines such as IGF-1.

We have continued to use the rat unloading model to produce skeletal hindlimb unloading of the growing rat. Bone formation was assessed by histomorphometry, ^{45}Ca and ^{3}H-proline incorporation, as well as change in fat-free weight and calcium content. Bone maturation was assessed by separating different fractions of powdered bone using toluene:bromoform density gradient centrifugation and evaluating the fractions for bone weight, total calcium, and ^{45}Ca and ^{3}H-proline incorporation. The effect of the active vitamin D metabolite $1,25(OH)_2D$ on bone formation and mineralization was determined by infusing the $1,25(OH)_2D$ into the rats with osmotic minipumps. Similar studies have been performed with growth hormone. The role of glucocorticoid hormones in mediating changes in bone formation with skeletal unloading was determined using surgical ablation techniques. To initiate studies of locally produced cytokines, we established cultures of mouse calvarial cells and long bone chondrocytes and plan to use such cultures to assess directly the effects of cytokines on bone cell function.

Accomplishments

(1) Demonstrated that high physiological doses of 1,25(OH)2D cause a mineralization defect as detected by density gradient analysis.

(2) Demonstrated that hindlimb unloading blunts the ability of growth hormone infusion to sustain growth of bone (tibia) in hypophysectomized rats.

(3) Demonstrated a decrease in somatomedin C (IGF-1) concentration in the tibial growth plates from unloaded animals compared to pair-fed controls.

(4) Demonstrated that hindlimb unloading does not result in a change in serum corticosterone level or its circadian rhythm.

(5) Demonstrated that adrenalectomy does not prevent the inhibition of bone growth that occurs in the tibiae of the hindlimb unloaded rat. Orchiectomy in combination with adrenalectomy was more effective than adrenalectomy alone in eliminating corticosterone production in the rats. This combination inhibited bone formation to such a degree that the additional effect of hindlimb unloading could not be discerned.

(6) Demonstrated that bone formation is accelerated when rats are allowed to recover after hindlimb unloading such that the deficit in bone mass is nearly completely reversed in two weeks.

(7) Demonstrated that fetal osteoblast cultures can be grown on collagen coated beads in the NASA Bioreactor. Under these conditions the cells lay down a collagen matrix and produce alkaline phosphatase.

Significance of the Accomplishments

Our current hypothesis is that the inhibition of bone formation that occurs transiently after skeletal unloading is due to a combination of systemic factors such as $1,25(OH)_2D$ and locally produced factors such as somatomedin C (IGF-1). Our observation that $1,25(OH)_2D$ infusion can lead to a mineralization defect encourages us to explore factors in bone under the control of $1,25(OH)_2D$ that regulate mineralization. Osteocalcin is the most obvious candidate. This bone matrix protein is increased by $1,25(OH)_2D$ and has been postulated to regulate bone crystal formation. In previous studies we have demonstrated that the concentration of osteocalcin in bone and blood falls during hindlimb unloading.

The decrease in somatomedin C levels in the growth plates of unweighted tibiae combined with the inability of growth hormone to reverse the inhibition of bone formation caused by unweighting suggests that the unloaded bone may have an abnormal response to growth hormone. This could be the key to understanding why bone formation is inhibited by unloading, since somatomedin C stimulates bone formation and its production by bone is thought to be under growth hormone control.

Our results indicating that adrenalectomy does not protect against the inhibition of bone formation by hindlimb unloading combined with our observations that corticosterone production is not increased by hindlimb unloading indicate that increased glucocorticoid production is not the reason bone formation is inhibited by hindlimb unloading.

The preliminary studies with osteoblasts in the Bioreactor point to future flight and ground-based opportunities to assess the effects of gravity on bone cell function.

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PHYSIOLOGY OF DEVELOPING GRAVITY RECEPTORS AND OTOLITH-OCULAR REFLEXES IN RAT

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Description of Research

The present study examines: (1) the physiologic responses of otolith afferents in the adult rat and during postnatal development, and (2) the otolith organ contribution to the vertical vestibulo-ocular reflexes (VOR).

During the past year, we have made progress in two areas. First, we have developed software for acquisition and analysis of spike train data from first-order otolith afferents. Second, we have characterized the physiologic responses of otolith afferents in adult rats. These efforts provide needed background data on peripheral otolith responses in the adult rat, which can then be compared with those in neonates to study the developing otolith system and with those in the CNS to examine central information processing in the otolith-ocular pathways. Such knowledge will permit an analysis on the effects of microgravity on the adult and developing mammalian gravity receptors.

Accomplishments

Our research accomplishments can be divided into two areas:

(1) Software development: Large amounts of spike train data must be processed to analyze the response of otolith afferents. Software development was continued during the past year to complete the routines for interspike interval histogramming and other display modes. All routines are written for use with existing IBM PC-XT and ILS analog-to-digital conversion software.

(2) Study of physiological responses of adult rat otolith receptors: Studies were conducted to characterize the physiologic responses of otolith afferents in the adult rat. Single unit recordings were recorded with glass micropipettes from first-order afferents within the internal auditory canal. Animals were anesthetized with chloral hydrate throughout the recording session. Once the units were isolated and distinguished from canal afferents by the absence of response to head angular acceleration in several head planes, they were subjected to several linear acceleration profiles: sinusoidal accelerations (0.025 to 1.0 Hz) in roll, and/or constant velocity (1^{o} - 10^{o}/sec), ramp changes of head position in roll extending $\pm$ 20^{o} from a normal erect head position. The superior and inferior divisions of the VIIIth nerve were sampled to observe the behavior of the utricle and saccule, respectively.

In response to ramp changes in head position, the vast majority of completely tested otolith units examined so far (29/34) show predominantly "tonic" responses in which the discharge rate is proportional to head position and is independent of the velocity of transition. The few remaining otolith units (5/34) have "phasic-tonic" responses characterized by an overshoot (or undershoot) during transition followed by a return, within 4-12 sec, to a new steady state rate. "Tonic" units show no dynamics, whereas the responses of "phasic-

tonic" units are proportional to both head position and the velocity of head transition. The proportion of "tonic" and "phasic-tonic" otolith afferents and their behavior in rat is similar to other mammalian species studied, e.g., cat (Anderson et al., 1978) and monkey (Fernandez and Goldberg, 1976).

The "tonic" units show a small phase lead (0-20° re. angular displacement) and approximately flat gain curves over the frequency range tested. On the other hand, "phasic-tonic" units show larger phase leads (20-55°) and 2-5 fold gain increases over the same frequency range. The linearity of the system was examined using a frequency of 0.25 Hz using head displacements of $\pm$ 2.5° to $\pm$ 25°, which corresponds to changes of $\pm$ 0.04 g to $\pm$ 0.39 g about the bitemporal axis, respectively. The response amplitude for each unit was found to be essentially a linear function of head displacement and independent of stimulus amplitude. Therefore, ***within the range of parameters used, the afferents respond in an essentially linear manner.***

Significance of the Accomplishments

These data from first-order otolith afferents in the adult rat will permit a quantitative comparison between peripheral and central parts of the otolith-ocular pathways, and will allow us to chart the postnatal stages in the physiological development of the peripheral otolith system. These data will also serve as control values with which to compare data from experimental animals reared on chemical or special diets to produce otolith deficits.

Publications

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BASIC GRAVITATIONAL REFLEXES IN THE LARVAL FROG

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Description of Research

As human beings on this planet, we have a basic spatial orientation with respect to what is up and what is down, even with our eyes closed and regardless of what our posture may be. This orientation is due to the ability of the inner ear endorgans to sense gravitational forces. How this information is processed by the brain to generate the spatial reference and gravitational reflex behavior is unknown. This study is directed towards understanding how the central nervous system of an extremely primitive vertebrate, the bullfrog tadpole, processes gravity sensing information in an attempt to uncover elementary principles of gravitational reflex organization, with the hope that a basic understanding of such organization in this primitive vertebrate may serve as a framework for understanding the more complex processes and reflex behavior found in humans and other mammals.

An important aspect of these larval frogs is the viability of the *in vitro* preparation of the isolated heads. Viability of this preparation is indicated by the presence of spontaneous, bilateral respiratory activity of the gills and mouth, by light-evoked, vibration-evoked, or spontaneously occurring arousal-like behavior and by the presence of compensatory eye movements and static eye deviation in response to tilt of the head. Another important aspect of this animal is that, since tadpoles have no arms or legs or much of a neck, nearly all gaze stabilization is accomplished with the eyes. This aspect gives the tadpole a very pronounced gravitationo-ocular reflex, which is uncommon in most vertebrates.

These two features, the viability of the isolated head and the high sensitivity of the eyes to head tilt, make this preparation extremely amenable to investigations of how neurons process gravity sensing information. Both physiological and anatomical techniques are employed in this study. Fine tipped electrodes are used to determine how particular cells respond to head tilt. Recordings from across individual cell membranes can determine the types of excitatory and inhibitory input that can influence the cells' activities during head tilt. Neuronal tracing techniques combined and in parallel with the physiological recordings can stain cells and cell populations that may be involved in these reflexes.

The ultimate goal of this study then is to determine the contributions of individual neurons to gravitational reflex behavior — What messages do they receive? How do these messages influence what they transmit? How does this activity influence gravitational reflex behavior?

Accomplishments

(1) The most significant accomplishment of the study to date is the ***development of an independent research laboratory by and for the principal investigator.*** This laboratory consists of some of the most advanced electrophysiological and anatomical equipment currently available, including an Axoclamp amplifier, a Nanostepper micromanipulator, a digitizing videocassette tape recorder, a Brown/Flaming electrode puller and electrode beveler, a Compaq 386/20 computer an Olympus differential interference contrast microscope, and a Vibratome 2000 sectioning system. This laboratory is one of the most highly advanced and is supported from this grant as well as an NSF grant.

(2) The high speed and capacity of the Compaq 386 computer allows digitization of the electrophysiological signals at a sample rate of 50,000 points per second for several minutes. The principal investigator has developed a software program that sequentially reads this information and through a complex algorithm is able to detect postsynaptic potentials and quantify features, such as onset time, amplitude, rise time, rate of rise, duration at half amplitude, and integral. This algorithm is generally applicable in that it has been applied to study a variety of synapses including the neuromuscular junction, the hair cell to afferent fiber synapse in the vestibular periphery, and the Mauthner cell to cranial relay neuron synapse of the goldfish. This algorithm is currently being utilized to study the hair cell to afferent fiber synapse (NSF study) and will be used in this study to distinguish postsynaptic potentials from different afferent sources.

(3) The establishment of this independent laboratory has allowed the performance of sustained experimentation. This opportunity has led to an increased viability (five days or longer) of the *in vitro* isolated head preparation. This increased viability permits anatomical tract tracing experiments to be performed, which allows for more precise experimental manipulation. The opportunity for sustained experimentation has also led to the refinement of surgical techniques. The vestibular labyrinth can now be opened while maintaining endorgan function, as evidenced by the persistence of vibrationo- and gravitationo-ocular reflexes and tilt sensitivity of central nervous system neurons. This procedure in the past resulted in the loss of such reflexive behavior (probably due to mechanical damage) and now permits the placement of stimulating electrodes on the VIIIth cranial nerve and its branches (in order to characterize inputs to a given cell), while maintaining, at least in part, normal reflex function.

(4) The neurons that control the eye muscles (i.e., the oculomotor neurons) are the current targets of the principal investigator's microelectrodes. ***Preliminary experiments indicate that the activity of these neurons can be modulated by lateral head tilt. In addition, electrical stimulation of either VIIIth nerve can activate these cells.*** Intracellular recordings reveal a complex sequence of excitatory and inhibitory post-synaptic potentials following such stimulation.

(5) In parallel to the above electrophysiological experiments, anatomical tract tracing techniques are being applied to this preparation to determine which cells project to the oculomotor neurons and which convey gravity signals. Initial experiments (involving transection of axons coursing to and through the oculomotor nucleus and horseradish peroxidase deposition on this lesion) indicate that many cells throughout the brain project into this region, most notably second order vestibular neurons and cells in the cerebellar peduncles. These experiments are being refined and coupled with the electrophysiology to understand the contributions of the individual neurons to the reflexive behavior.

Significance of the Accomplishments

The established laboratory incorporates the latest, "state-of-the-art," high technology, which maximizes the chances for successful and high quality experimentation. The accompanying software development extends the capabilities of synaptic potential quantitation not heretofore practical or even possible. Such quantitation, which previously took weeks by hand and days by older computers, now takes only a matter of hours. These developments, in terms of the establishment of a laboratory and analytical software, have provided the opportunity for sustained experimentation. This opportunity has in turn led to an increase in the viability of the isolated head, allowing for more refined and flexible experimentation. Initial, successful electrophysiological characterization of oculomotor neurons and accompanying successful anatomical tracing techniques revealing the source of afferents to these neurons demonstrate the practicability of the isolated head for this study.

In short, the accomplishments of this year pave the way for future experiments aimed at determining the role of individual neurons in the mediation of gravitationo-ocular reflexes, a necessary step in determining the organization of the neuronal networks that accomplish gravitationo-ocular reflex control in this primitive vertebrate.

STUDIES OF INTERCELLULAR COMMUNICATION AND INTRACELLULAR RESPONSES BY BONE CELLS TO SIMULATED WEIGHTLESSNESS

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Description of Research

The cells of bone are unique in the variety of functions which they can express. They are responsible for the synthesis of the organic matrix of bone, then they mineralize it with a Ca-PO_4 hydroxyapatite, and finally they can remodel the overall structure according to the mechanical forces applied to the bone. Our interests include all of these functions but in the reverse order. In other words, how do bone cells "sense" mechanical force and respond to it? The absence of mechanical force is seen in the extreme during spaceflight and hypogravity. Previous studies on rats which have experienced hypogravity have shown a loss of bone mass due to a reduction in new bone formation.

We are using a combination of techniques to study bone cells and the physiology of the skeleton following actual spaceflight. A significant portion of our research included the preparation for and the actual study of skeletal tissues from the 12.5 day Cosmos 1887 biosatellite flight. We used a combination of light and electron microscopic and morphometric techniques to determine cellular changes which occurred to the bone forming cells.

In addition to the Cosmos 1887 studies, we have continued our collaboration with Dr. Emily Morey-Holton at NASA Ames Research Center on the growth of bone cells on a three dimensional lattice made of beads. These beads have been coated with different substances (e.g., collagen) to determine the effect on cell growth and differentiation. In addition, particles of bone have been added to some cultures to determine how cells adhere and grow on these more natural substances.

Accomplishments

(1) ***Spaceflight affected the blood vessels within compact bone.*** Following spaceflight there was structural damage to the endothelial cells and a reduction in some enzyme activities.

(2) ***There was an asymmetrical distribution of damaged vessels in the compact bone*** (Figure 1). The damage appeared in the outer one-third of the long bones. No damage was noted in the marrow or the vessels at the growing ends of the long bones.

(3) ***Methods have been developed to determine the energy state of the bone cells.*** The relative determination of mitochondrial activity and the quantitation of "shuttle" vesicles in the Golgi complex will provide evidence of the energy state of the cells under investigation.

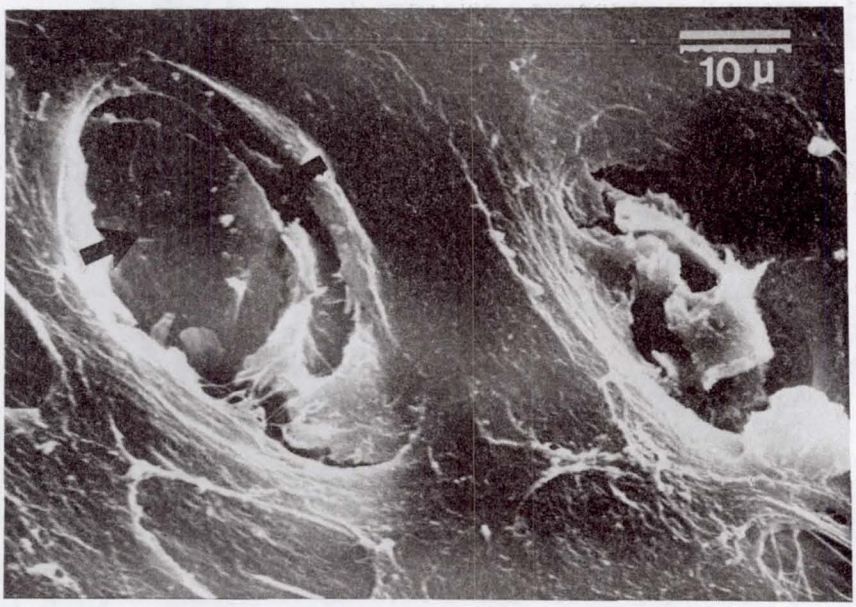

Figure 1. A scanning electron micrograph of two blood vessels in compact bone. Note that the vessel on the left contains an amorphous mass (arrows) of material which could prevent blood flow through the vascular space. Magnification = 1,500X.

(4) ***Bone cells grown in culture on a three dimensional structure appear to differentiate more rapidly than those grown on a standard flat surface.*** We are continuing to alter the environment of these cells to determine how they respond to various environmental factors.

Significance of the Accomplishments

It was surprising to find that spaceflight altered the morphology and enzymology of specific vessels within the dense weight-bearing long bones (accomplishment #1). The prevalence of vascular damage along the outer one-third of the long bone (accomplishment #2) can be explained by the unusual vascular arrangement in bone. The blood flow in bone is in a centrifugal pattern with blood flowing from the marrow cavity towards the outer periosteal surface. This flow also depends on some muscular contraction to help the venous drainage away from the bone. The loss of muscular tone and the cardiovascular-deconditioning which occurs during spaceflight may contribute significantly to the vascular changes seen in the long bones.

Any alteration to the blood flow in bone will also significantly affect the metabolic activity of the affected blood vessels and the adjacent bone cells which depend on those vessels. We are developing methods to determine the energy levels of individual bone cells (accomplishment #3) to determine which cells in bone are affected by hypogravity and spaceflight.

As a model system to mimic what is found in whole animal studies, there is an ongoing effort to culture bone cells (accomplishment #4) and determine what factors are critical for

cellular differentiation and function. What we learn in this simplified system will be compared to our studies of bone in whole animals.

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GROWTH AND DIFFERENTIATION OF MAMMALIAN EMBRYONIC TISSUES EXPOSED TO HYPERGRAVITY *IN VIVO* AND *IN VITRO*

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Description of Research

The purpose of this research is to determine the effects of excess gravity on development of the mammalian embryo *in utero* and of mammalian tissues in culture. The use of excess gravity enables us to identify systems and tissues that are sensitive to gravitational changes, and which therefore might be affected by exposure to the microgravity environment of space. The use of *in vivo* and *in vitro* systems allows us to distinguish any direct effects on the embryo from indirect effects resulting from changes occurring in the mother.

Accomplishments

(1) The ability to do timed matings on the centrifuge allowed us to rear three generations of animals at 2.3 and 2.6 g.

(2) Observations of the behavior of these animals indicated that they were less sensitive to a decreased gravitational field, i.e., return to 1 g Earth gravity, than were animals that were placed on the centrifuge at four weeks of age.

(3) Weights of these animals were again less than the weights of controls.

(4) In a repeat of this experiment, several litters born at excess g are now being reared.

(5) Samples of adult animals were taken as follows:

a. Dr. A. Cogoli made spleen cell cultures to examine lymphocyte activation, fixed bits of spleen for EM, and labeled spleen cells for cell sorting.
b. Skulls, which are part of an ongoing cephalometric study, are being analyzed at Baylor College of Dentistry in Dallas.
c. Lungs were perfused for further morphometric studies by Dr. Weibel of the Anatomisches Institut Universitat Bern. Numerous other tissue samples were taken for analyses by other people at the Institute.
d. Long bones were fixed for morphometric analyses in this lab.
e. Gastrocnemius and masseter muscle were used by Dr. Max Hutchins of the University of Texas Dental Branch physiology department for EMG studies.

(6) Observations funded by this propsal were responsible for our being able to predict results for SL3 and the Cosmos biosatellite flights in 1987 and 1989. These results also led to proposal for the "CELLS" experiment which is manifested for IML-1 and which will be the first culture of skeletal cells in space.

Significance of the Accomplishments

Finding #1: To analyze effects of any environmental change on development, it is necessary to be able to carry out timed matings, and the ability to do that on the centrifuge will be useful for centrifuges on space station and shuttle.

Finding #2: Although we were not able to fully measure these behaviors, the observations were noticeable and the changed behavior of these mice show that some change in the neurovestibular system occurred.

Finding #3: Small they may be, but mice are still affected by gravitational changes.

Finding #4: This will enable us to compare effects on rats with effects on mice, and to see if the effects of μg are related to the effects of excess g.

Finding #5: The significant sample sharing in this experiment (and all previous ones) shows the widespread interest in centrifugation, and the intuitive knowledge, based on physical principles, that gravity and its effects form a continuum.

Finding #6: We have made predictions about cartilage development in space as a result of these studies. These are supported by more data than any clinostat experiment involving animals or animal cells.

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ION CHANNELS IN STATOCYST HAIR CELLS OF *HERMISSENDA CRASSICORNIS*

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Description of Research

Our goals remain the identification and characterization of ion channel families which mediate mechanosensory transduction, amplification, and integration in the hair cells of the statocyst organ of the marine snail *Hermissenda*. We have utilized conventional two-microelectrode voltage clamp, fluctuation analysis of membrane current noise, and single-channel patch clamp methods to study several classes of ion channels in *Hermissenda* hair cells. These include: (1) mechanosensory-activated ion channels, whose probability of opening increases when the hair cells are gravitationally stimulated; (2) three different classes of voltage-dependent K channels, which are activated when the hair cell is depolarized as a result of mechanosensory stimulation; and (3) a voltage-dependent Ca current.

Accomplishments

(1) Fluctuation analysis of ionic current noise of hair cell membranes, under voltage-clamp control, indicates that gravitational stimulation of hair cells opens mechanosensory-activated ion channels. The contribution of mechanosensory-activated channels to current noise was maximized by clamping the cell to negative membrane potentials ($\leq$ -60 mV) at which the probability of voltage-dependent channels being open was quite low, and in some experiments by the addition of inhibitors of voltage-dependent K channels to the bath. Current-noise amplitude was increased by 1-2 orders of magnitude when the hair cell was mechanically stimulated by tilting the statocyst so that the statoconia pushed against the motile cilia. ***Current noise amplitude of mechanically stimulated cells: (a) increased in approximately linear fashion as the holding potential was made more negative than -30 mV, (b) was greatly reduced by removal of extracellular Na ions from the bath, and (c) was blocked by 1-2 μM concentrations of gadolinium ion, an inhibitor of stretch-activated mechanosensory ion channels in other systems.*** Spectral density functions (SDFs) of current noise were non-regular and could be well-fit by double Lorentzian functions (Figure 1). In many cells, the SDFs were characterized by a hump in the 7-12 Hz range, which corresponds to the fundamental beat frequency of the motile cilia. ***Fluctuation analysis yielded a single-channel conductance of ~40 pS, when Na was the main permeant charge carrying ion, and an estimated 200-300 channels per hair cell. The channel is relatively non-selective for cations, however, and also allows both Ca and K to pass.***

(2) We have continued our characterization of the three voltage-dependent K channel families in some membranes of hair cells. We have begun to construct Hodgkin-Huxley type models for each, as well as a computational model of how the K currents and the current through the mechanosensory-activated ion channels combine to determine the integrative response of the hair cell to sustained mechanical or electrical stimulation. The kinetics of the "A" current have now been extensively studied. This transient K current

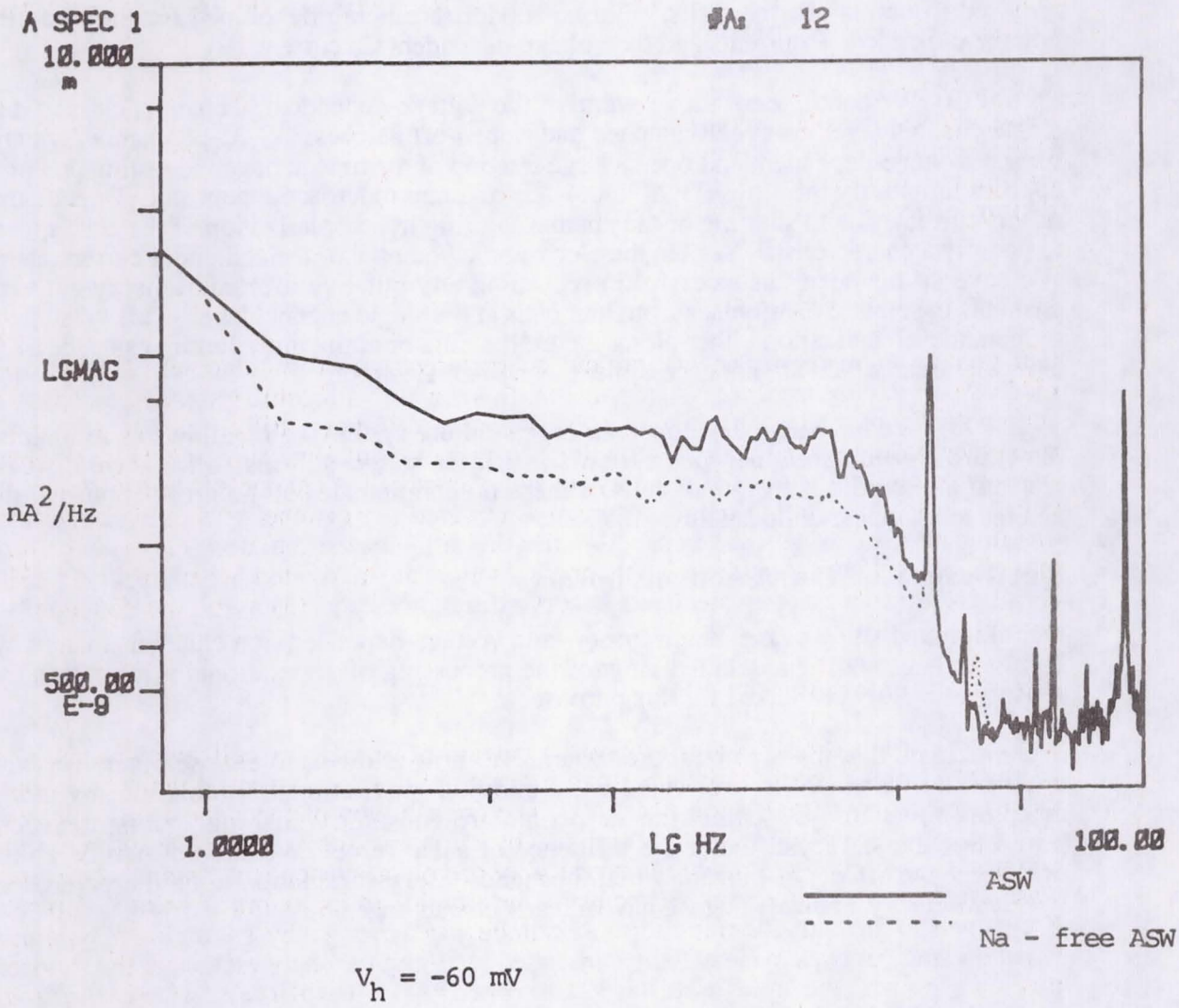

Figure 1. Spectral density function (SDF) of ionic current noise from voltage-clamped *Hermissenda* statocyst hair cell membrane, subject to tonic mechanosensory stimulation. Power spectrum amplitudes recorded from the cell in normal artificial seawater (430 mM Na ion concentration) is greater than that recorded when Na was removed and choline ions were substituted. This indicates that the mechanosensory-activated channels allow Na to pass. Cell was voltage-clamped at -60MV to minimize the contribution of K and Ca channels to the current noise. Power spectra computed from 25 sec duration records of current noise, filtered at 300 Hz.

shows sigmoidal activation characteristics, typically peaking within 5-10 msec following a command depolarization (Figure 2). Data from the rising phase of the current were fit to the activation component of a Hodgkin-Huxley type model, with third order kinetics, and time constants ranging from 0.8 - 1.5 msec were extracted (20 C). These time constants were largely independent of voltage. Relaxation of the "A" current was well fit by a single exponential decay function with time constants ranging from 20 - 160 msec, depending upon the cell and membrane potential. In some cases, double exponential functions were

required to obtain adequate fits. Similar models are being developed for the two other voltage-dependent K currents and the voltage-dependent Ca current.

(3) We continue to study several of the voltage-dependent K channel classes at the single channel level, from cell-attached and ripped-off patches. A ~35 pS channel which is weakly voltage-dependent and open a large fraction of the time at negative resting potentials appears to underly the slow, TEA- and 4-AP-resistant macroscopic current. Deactivation of this current, due to shutting of the channels during hyperpolarization of the cell, appears to underly a characteristic "sag" in the electrotonic potential of the cell under current clamp. We have so far been unsuccessful in recording any putative mechanosensory-activated channels in somatic membranes from hair cells at the single channel level. This presumably reflects their localization either along the motile cilia or at the inner luminal surface of the hair cell, areas which are not accessible to our patch electrodes.

(4) We have also developed an organ culture system which allows us to maintain the entire *Hermissenda* nervous system, or just the statocyst, *in vitro* for several weeks. Our initial recordings from hair cells of these preparations reveal no gross abnormalities, and the cells appear quite healthy.

Significance of the Accomplishments

Our continued study of mechanosensory- and voltage-dependent ion channels in statocyst hair cells is providing a detailed view of the processing of gravitational information by a sensory receptor specialized for this purpose.

We anticipate that the organ culture system will now allow us to expose otherwise intact and normal hair cells to prolonged and abnormal gravitational stimulation, by simply orienting the statocyst so that the statoconia are constantly pushing against a selected portion of the statocyst, under the influence of a 1 g force. We should then be able to document the effects of this abnormal mechanosensory stimulation upon the voltage and mechanosensory channels that we have already characterized.

KINETICS OF "A" CURRENT IN STATOCYST HAIR CELL OF *HERMISSENDA*

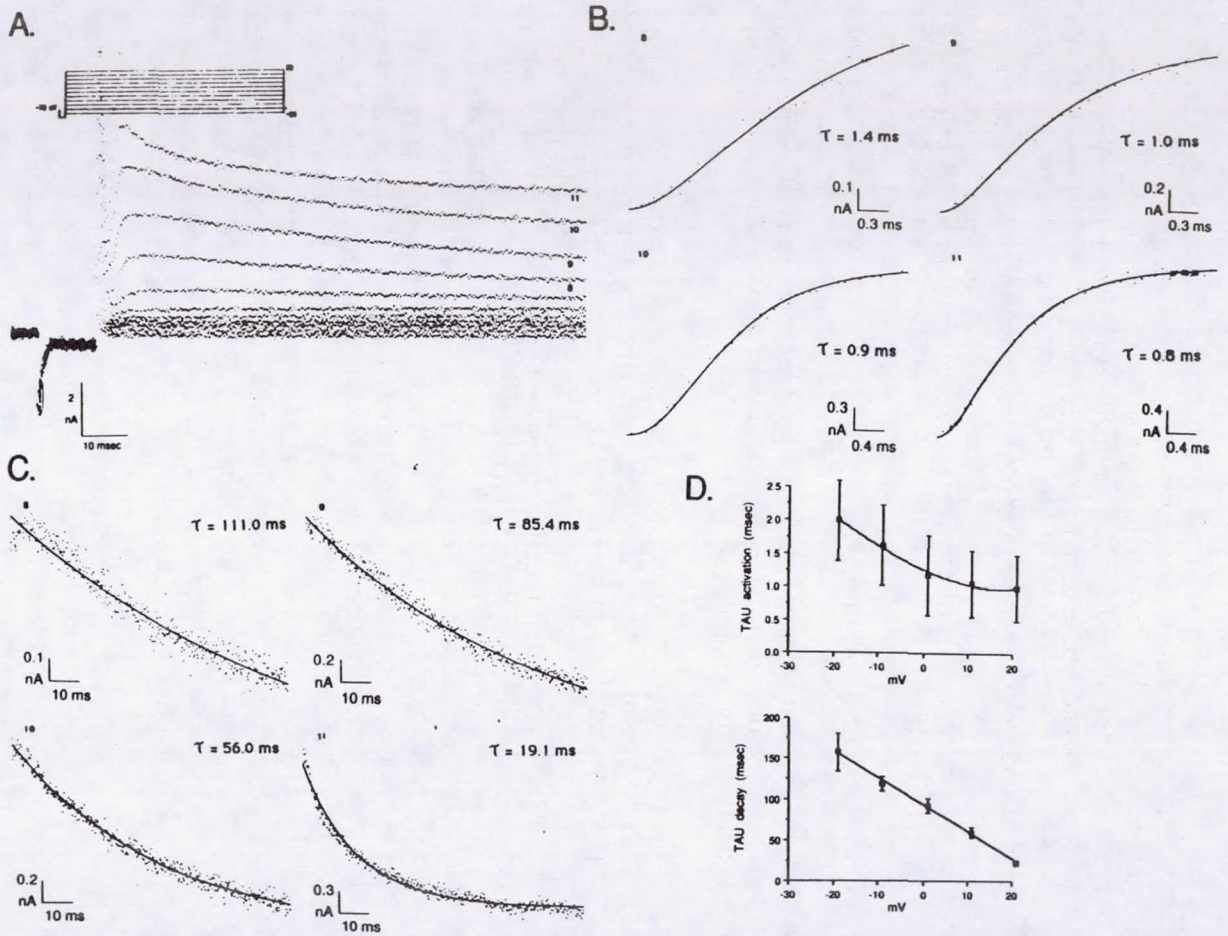

Figure 2. Kinetics for activation and inactivation of "A" type voltage-dependent K current of hair cell. (A) Family of outward currents elicited by 50 msec depolarizations to membrane potentials indicated in the pulse-protocol insert (top left). Trace numbers 8-11 identify current traces for which activation- and decay-time constants were extracted in parts B & C. (B) Predicted and observed current traces for rising phase of "A" current for cell whose records appear in part A. Current traces were fit to a Hodgkin-Huxley model, with third-order kinetics. Time constants (tau's) show a slight reduction as membrane potential becomes more positive. (C) Predicted and observed current traces for decay of "A" current. Currents were fit to a single-exponential decay function. Time constants (tau's) are markedly voltage-dependent, showing large decreases as the membrane potential becomes more positive. (D) Average activation- and decay-time constants for "A" currents from eight different cells. Values are mean +/- SEM. Decay time constant was markedly voltage-dependent, while that of activation was not (note change in scale).

INFLUENCES OF SYNAPTIC GENESIS ON THE ARCHITECTURE OF VESTIBULAR EPITHELIA

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Description of Research

The objective of this project is to study the vestibular epithelia of the utricle and saccule in order to: (1) delineate modifications of cells known in other organs to relate to synaptic genesis, and (2) investigate the relationship that might exist between refinement of the epithelia and changes in the formation of the statoconial membrane.

The maculae are gravity detectors and their development should be influenced by the microgravity environment. Thus, it is essential to understand normal changes of the maculae in ground-based experiments so that inflight experiments can be evaluated and justified. Since the statoconial membrane is thought to provide necessary load for deflection of cilia that result in the stimulation of hair cells, knowing whether the epithelia and the statoconial membrane develop concomitantly, or if one of them precedes the other, is important.

Accomplishments

Figure 1 shows a diagrammatic representation of the main changes occurring to the vestibular epithelia of the chick inner ear. Cuboidal epithelial cells found in the otic vesicle quickly transform by the third day of incubation into pseudostratified epithelia. The basement membrane becomes defined, and afferent dendrite penetrate the basement membrane in search of a target to innervate. ***Eventually, the growth cones of the afferent fibers encounter undifferentiated hair cells and subsequently final innervation patterns are established.*** That is, Type I hair cells are encased by a nerve chalice while Type II hair cells remain innervated by bouton-like ending. The sequence of events just described occurs in a very timely fashion. By stage 25, 4 days of incubation, the cells are actively engaged in the production of materials needed for growth and cell division. This is exemplified by the large amount of free ribosomes and heterochromatic nuclei as well as dense cytoplasm. By six days of incubation, or the beginning of the second trimester of gestation in the chick, the first hints of differentiation between hair cells and supporting cells are apparent. For instance, the cytoplasm of supporting cells becomes less dense while that of the hair cells become more dense. In addition, supporting cells lose their kinocilia while hair cells enlarge their stereocilia.

The changes described above have been derived from ultrastructure observations of micrographs at different development stages. For illustration purposes, in Figure 2, portions of the hair cells (H) and afferent fibers are shown. Arrows point to the various places where the growth cone is contacting the hair cells making partial synaptic contacts. ***A darker contact of cell membranes (upper arrow) is opposed to a dense body which is called synaptic ribbon. This synaptic ribbon,*** or synaptic body, has been taken as an ***indicator of mature afferent function at the ultrastructure level.*** In Figure 3, a higher magnification of a different synaptic contact from another hair cell better illustrates the appearance of the synaptic body as well as the opposing membrane between the growth cone of the afferent fiber and the hair cells. The arrow is pointing to a well formed synaptic vesicle that is directly connected to the synaptic bar. The

transition between the appearance of the structure shown in Figure 2 and that of Figure 3 is accompanied by myelination of the dendritic fibers as well as completion of the statoconial membrane resting on the epithelia.

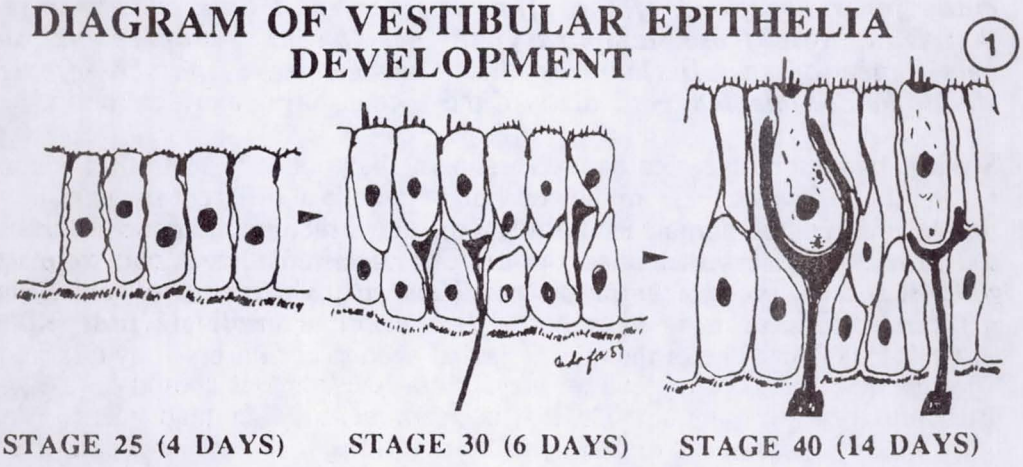

Figure 1. Diagram of vestibular epithelia development showing transformation of an undifferentiated cuboidal epithelia into pseudostratified epithelia by Stage 30. Rudimentary, yet well formed, vestibular epithelia with Type I and Type II hair cells are present by Stage 40.

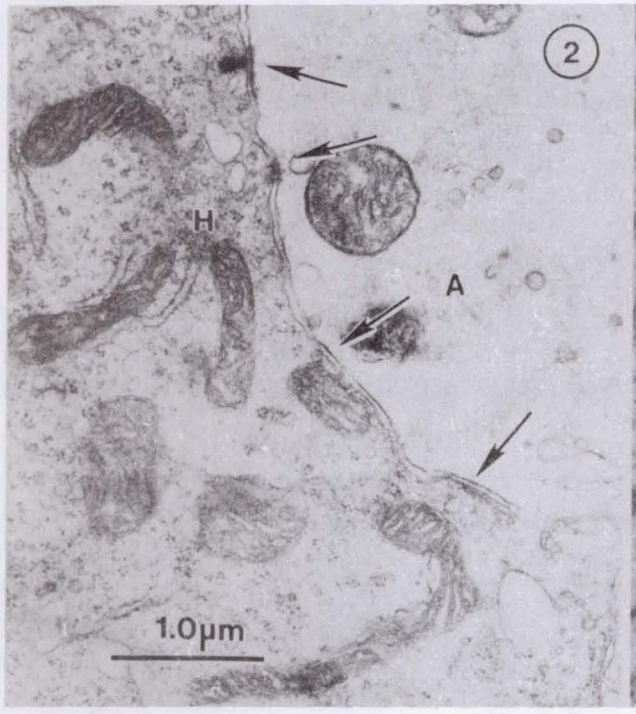

Figure 2. Electron micrograph showing portions of the hair cells (H) and portion of a growth cone from an afferent fiber (A) indicating point of contact between the fiber and the hair cells as shown by the arrows. The upper arrow is showing a density of the membrane opposed to a dark synaptic bar inside the hair cell.

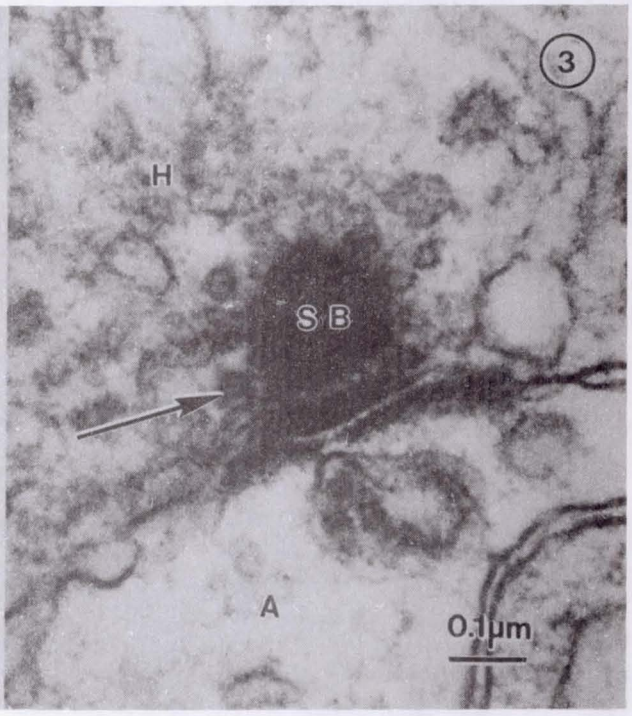

Figure 3. The synaptic bar (SB) is found in the presynaptic target, in this case the hair cell (H), and eventually becomes encircled by many afferent synaptic vesicles as can be seen by the arrow. Notice the well developed presynaptic density opposing the afferent fibers (A). Opposition of this type of ultrastructural feature and an increased number of synaptic vesicles are usually recognized as functional afferent synapses in adults and near term embryos.

Significance of the Accomplishments

It is possible that development of the hair cells and afferent fibers in the vestibular epithelia is similar to that reported previously for the cochlear hair cells. If that is the case, then ***many transformations of the afferent fibers and hair cells themselves during the transition from temporary to permanent synapses as well as the persistence of certain features that characterize a mature afferent synapses should be documented,*** particularly if they occur during a critical period.

Studies by other investigators have shown that, indeed, vestibular responses can be recorded non-invasively from the vestibular epithelia at different stages of development. It will be of utmost importance to correlate the ultrastructure maturation of the epithelia with the onset of those vestibular responses. For instance, ***are rudimentary responses obtained from young embryos modified at the time that myelination of the afferent fibers occurs, or are the two events completely independent of each other?*** This is just one of the questions that need to be addressed by this study.

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HOMEOSTASIS IN PRIMATES IN HYPERACCELERATION FIELDS

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Description of Research

The ultimate goal of this research is to understand the physiological influence of gravity on living organisms, particularly mammals. Of particular interest are the physiological mechanisms leading to adaptation of an organism to an altered gravitational environment. Included in these responses are identification of receptors, pathways of information transfer (neural and endocrine), mechanisms of integration of information, and pathways and mechanisms affecting the organism's response to alterations in gravity.

The adaptation of homeostatic systems to changes in gravity are poorly understood. Such changes in centrifuged animals include depressed body temperature, alterations in circadian timekeeping, and changes in the level of arousal. To date, research interests in this laboratory have focused on the sensitivity of these and other homeostatic systems to alterations in gravity. This research has provided both a demonstration of the responsiveness of these systems to gravity as well as efforts to elucidate the underlying mechanisms of the responses. Further, this program has focused on the responses of the whole organism to further understand the interaction between the various physiological systems of interest. This research has required the ability to alter the dynamic environment of the organism. At the Chronic Acceleration Research Unit at the University of California at Davis, four centrifuges (8 to18 ft in diameter) are available. These facilities provide for acute and chronic exposures of animals to fields ranging from 1 to 20 g.

Accomplishments

(1) The circadian rhythms of heart rate and activity in rats were examined in a 2 g hyperdynamic field via chronic centrifugation. ***In a normal 12:12 light/dark cycle, rats exhibited reductions in the amplitudes of heart rate and activity rhythms following exposure to 2 g.*** Rhythm amplitude returned towards normal 7-8 days later for activity and 10-12 days later for heart rate. However, during the period of 2 g exposure, the circadian rhythm amplitudes never returned to control levels.

(2) The recovery of body temperature circadian rhythm following exposure to a 2 g hyperdynamic environment was examined under different lighting conditions. ***Following exposure to 2 g, rats in a 12:12 light/dark cycle exhibited recovery of body temperature rhythm amplitude earlier than rats in a constant light environment*** (10 vs. 21 days, respectively).

Significance of the Accomplishments

Our previous studies demonstrated that exposure to a 2 g hyperdynamic field via centrifugation resulted in a severe reduction in temperature rhythm amplitude. Finding #1 extends these observations to other physiological systems (heart rate and activity). The reduced circadian amplitude in several physiological variables suggests that the central pacemaker that controls circadian rhythmicity may be affected directly by the altered gravitational field. A depressed circadian pacemaker could result in serious deficits in an

animal's ability to respond to environmental challenges such as extreme cold during this recovery period.

Finding #2 demonstrates that 12:12 light/dark (LD) cycle can mask the recovery of circadian rhythms. Even though temperature rhythms return after 10 days in a normal LD 12:12 cycle, the underlying circadian pacemaker may still be depressed. Therefore, the imposed environmental LD cycle has masked the actual recovery. It will be important to determine whether this masking effect can minimize the potential deficits of an animal's ability to respond to an environmental challenge. Such a possibility stresses the importance of a light/dark cycle in an artificial environment such as spaceflight.

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NEURAL MECHANISMS BY WHICH GRAVITATIONAL STIMULI AND STRESS AFFECT THE SECRETION OF RENIN AND OTHER HORMONES

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Description of Research

The long-term goal of this research is delineation of the brain pathways and neurotransmitters that mediate gravitational and stress-induced changes in the secretion of renin and other salt and water-regulating hormones. Evidence from this laboratory indicates that stimulation of certain serotonin-secreting neurons in the dorsal raphe nucleus of the midbrain increases renin secretion, and that these neurons project to the hypothalamus. One of our goals has been determination of the specific parts of the hypothalamus that affect circulating renin and angiotensin, and the importance of this region in the physiological control of renin secretion. Another goal has been determination of the pathways from the nervous system to the renin-secreting cells in the kidney. To study the role of the hypothalamus and other parts of the brain in the regulation of renin secretion, we have used stimuli which increase renin secretion in diverse ways. Emphasis has been placed on the postural stress of 45° head-up tilt. Other standard tests include: (1) administration of the serotonin-releasing drug p-chloroamphetamine (PCA); (2) the psychological stress of immobilization; (3) the chronic volume depletion stress of a low sodium diet; and (4) the acute volume depletion stress of nonhypotensive hemorrhage. We have also begun to study the role of the vasopressin-secreting neurons that connect the hypothalamus to the brain stem and spinal cord in the regulation of renin secretion.

Accomplishments

(1) ***Demonstration that lesions of the ventromedial nuclei of the hypothalamus reduce the increases in plasma renin activity (PRA) produced by PCA, immobilization, head-up tilt, and a low sodium diet.*** These lesions do not reduce circulating angiotensinogen, and the effect of the ventromedial nuclei is presumably via the renal nerves.

(2) Demonstration that like the PRA response to PCA, ***the renin responses to immobilization and head-up tilt are blocked by the ß-adrenergic blocking drug propranolol.*** This indicates that in all three situations, ***the final common pathway from the spinal cord to the renin-secreting cells is sympathetic.*** We have also investigated the effect of propranolol on the increase in PRA produced by a low sodium diet. PRA is lower in propranolol-injected rats fed a low sodium diet than it is in vehicle-injected controls on the same diet. However, there is a defect in these experiments. Injection by itself is a stress, and it is not possible to say whether propranolol is simply blocking the response to injection stress or reducing the overall response to sodium depletion. During the past year, we have perfected the technique of chronic implantation of catheters in conscious rats and will repeat the propranolol experiment in rats with a low sodium diet without the stress of holding the rats and injecting them.

(3) Our evidence indicates that in addition to regulating angiotensinogen secretion, the ***paraventricular nuclei of the hypothalamus are involved in the regulation***

of the secretion of renin from the kidneys in some situations. Since some of the paraventricular neurons secrete vasopressin into the circulation from the posterior pituitary and others project to neurons regulating cardiovascular function in the brain stem and spinal cord, we have begun to explore the relationship of vasopressin to renin secretion. Systemically administered vasopressin inhibits renin secretion, possibly by a direct effect on the renin secreting cells in the kidney and possibly by a baroreceptor reflex. In addition, the vasopressin secreted by neurons in the brain stem or spinal cord could inhibit renin secretion. In studies using homozygous Brattleboro rats that due to a genetic defect have no vasopressin in their brains or circulating blood, we found that ß-adrenergic blockade produced by propranolol lowered circulating renin to essentially normal values. This rules out the withdrawal of a direct inhibitory effect on the renin-secreting cells as the cause of the renin hypersecretion and indicates that the Brattleboro rats have chronically increased sympathetic discharge. In another set of experiments, we used osmotic minipumps to infuse vasopressin subcutaneously in Brattleboro rats in a dose which restored circulating vasopressin to normal. The increased renin secretion was unaffected even though urine volume and water intake were markedly reduced and the concentration of the urine was increased. This seems to rule out a reflex originating peripherally. However, since very little vasopressin penetrates the blood-brain barrier, the increased sympathetic discharge that produces the renin hypersecretion could be central in origin. Therefore, we continued the experiments started by Dr. Gotoh in which vasopressin was injected directly into the brain. In addition, one should be able to reduce circulating vasopressin to zero without affecting brain vasopressin by simply removing the posterior lobe of the pituitary gland, the site where vasopressin is secreted into the circulation. If our hypothesis is correct, these animals should have normal or nearly normal renin secretion.

(4) We have also done considerable work on brain regulation of angiotensinogen secretion during the past year. As noted before, we originally discovered that lesions of the paraventricular nuclei decreased circulating angiotensinogen. Since thyroid hormones, adrenal glucocorticoids, and estrogens are known to increase the secretion of angiotensinogen from the liver and the paraventricular nuclei are involved in the regulation of the secretion of thyroid hormones and glucocorticoids via hypothalamic control of the secretion of TSH and ACTH by the anterior pituitary gland, we compared the effect of hypophysectomy with the effect of paraventricular lesions at various times while we also measured circulating triiodothyronine, thyroxine, ACTH, and LH. Hypophysectomy caused a slowly developing decrease in circulating angiotensinogen which mirrored the decrease produced by paraventricular lesions. In both instances, there was a prompt decrease in circulating thyroid hormones. Hypophysectomy also decreased plasma ACTH, but paraventricular lesions, as reported by others, only abolished stress-induced increases in ACTH secretion and failed to affect resting ACTH. The lesions also had no effect on plasma LH. Thus, ***it seems likely the decrease in circulating angiotensinogen is due to decreased thyroid secretion.*** One pilot experiment with replacement of circulating thyroid hormones in lesioned animals has been carried out and others are planned in the near future.

(5) In the course of the research on paraventricular lesions, we also observed that in six rats, lesions anterior to the paraventricular nuclei decrease circulating angiotensinogen without affecting plasma triiodothyronine, thyroxine, ACTH, or LH. The lesions in each of these rats have now been mapped by projecting the sections on paper, and the common area of destruction in them is the anterior end of the third ventricle, including the organum vasculosum of the lamina terminalis and the median preoptic nucleus. These structures are known to be involved in the regulation of sodium and water excretion. We will now proceed to make specific lesions in this region to confirm our initial finding, and provided it is confirmed, we will investigate the mechanism involved in detail.

(6) In the course of the experiments described in the preceding paragraph, we made the chance observation that 24 hours after surgical stress and some anesthetics, plasma angiotensinogen was elevated with little if any change in PRA. This could be due to the fact that stress causes a transient increase in circulating glucocorticoids which have a long lasting effect on angiotensinogen secretion. This possibility will be explored during the coming year.

Significance of the Accomplishments

The experiments described above and experiments conducted in previous years have done much to elucidate the role of the brain in the regulation of renin secretion. This has appreciable significance for NASA because both postural changes and stressful stimuli increase renin secretion and renin plays a vital role in salt and water balance and the maintenance of blood pressure. In addition, our demonstration that brain lesions lower circulating angiotensinogen is important because it demonstrates for the first time that there is a neuroendocrine and possibly a neural mechanism regulating the circulating level of this important component of the renin-angiotensin system.

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VECTOR-FREE GRAVITY INTERFERES WITH SYNAPSE DEVELOPMENT

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Description of Research

Prolonged spaceflights and plans for the colonization of space have opened two new areas of research in biology. The first concerns the well-being of developing biological systems in flights of even short durations. The second concerns the possibility of investigating, from an evolutionary perspective, the contribution of gravity to development.

Our objective is to examine, in-depth, specific aspects of nervous system development under simulated microgravity to gain a better understanding of how gravity influences ontogenetic development of the brain while at the same time exploring the possibility that the constancy of gravity, on Earth, has contributed significantly to cellular evolution.

From the data that we have gathered over the past 4 years, we conclude that cellular processes are profoundly affected when cells are grown in vector-free gravity. We believe that this environment provides a faithful simulation of weightlessness, and therefore suggests that during embryonic development a window of sensitivity exists during which cells are vulnerable to alterations in the gravitational field. Specifically, we show that vector-free gravity interferes with the process of nerve-induced receptor accumulation during synapse formation. Recently, we have started looking for the underpinnings of the alterations to nerve-induced receptor patching by monitoring the distribution of cytoskeletal proteins. With this approach we are probing changes in the structure of the cellular scaffolding responsible for anchoring of proteins to specific locations in the cell subsequent to growth of cells in vector-free gravity.

Accomplishments

In an earlier report we provided information on morphologic changes which occur in embryonic nerve and muscle cells grown in culture under conditions of vector-free gravity. We have now extended these findings to a very specific process which occurs normally during synapse development: the accumulation of receptors, for the neurotransmitter acetylcholine, to the junctional zone between these two cells.

Accumulation of acetylcholine receptors (AChRs) to the synaptic zone is known to be a process induced by signals which originate from the nerve cell. The nature of the signals is still under scrutiny but is likely to involve a combination of contact between the two cells and the secretion of a soluble factor by the nerve. This process is essential to brain function and to the control of peripheral tissues by the brain. It provides for effective, intercellular chemical transmission by the insertion of receptors in the postsynaptic cell near the transmitter release sites in the presynaptic cell.

Our results show that ***development in a slow clinostat (1-10 rpm) results in dramatic reductions of the appearance of nerve-induced AChR accumulation at the point of contact between nerve and muscle cells in culture*** (Figures A-F). This reduction is most notable if innervation takes place during the period of rotation in

the clinostat. If synapse formation takes place prior to rotation, the preexisting receptor patches are much more resistant to this environmental perturbation.

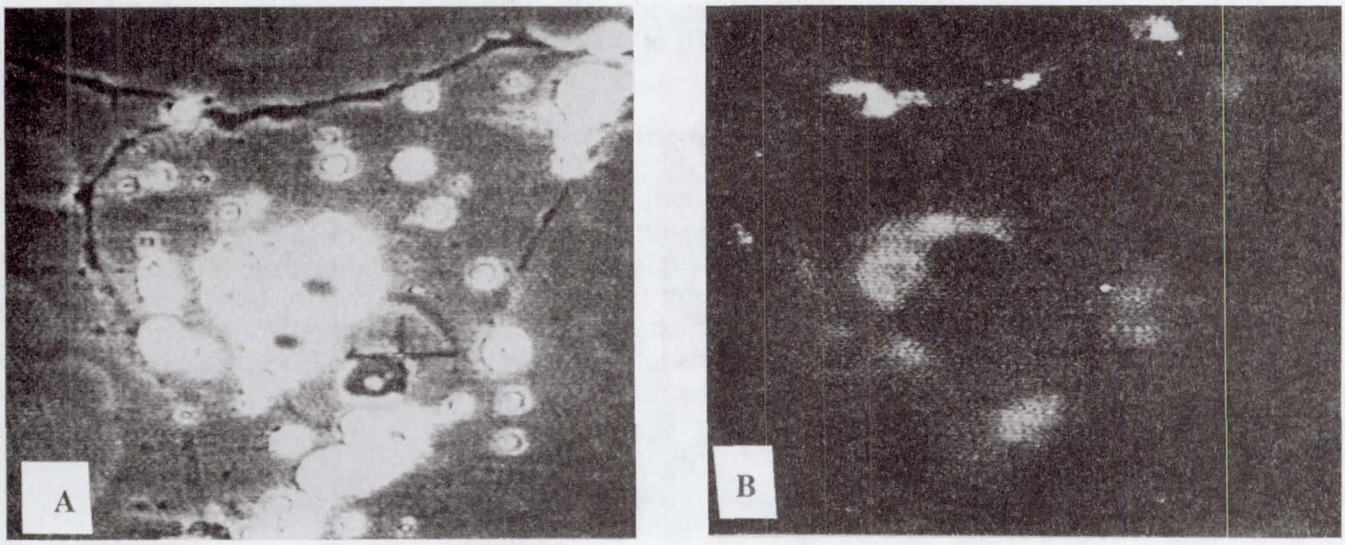
We have carried out an extensive series of control experiments in order to reduce the possibility that rotation of cells in the clinostat causes damage to the cells simply because of shearing forces, the loss of contact between nerve and muscle, or the accelerated diffusion of a putative inducing factor secreted from the nerve cells. To this end, we rotated cultures around a vertical axis, subjected cells to an oscillating platform, grew cells in inverted cultures, and subjected cells to increased hydrostatic and centrifugal forces. Receptor accumulation in these cultures was not statistically different from that occurring in stationary controls.

Significance of the Accomplishments

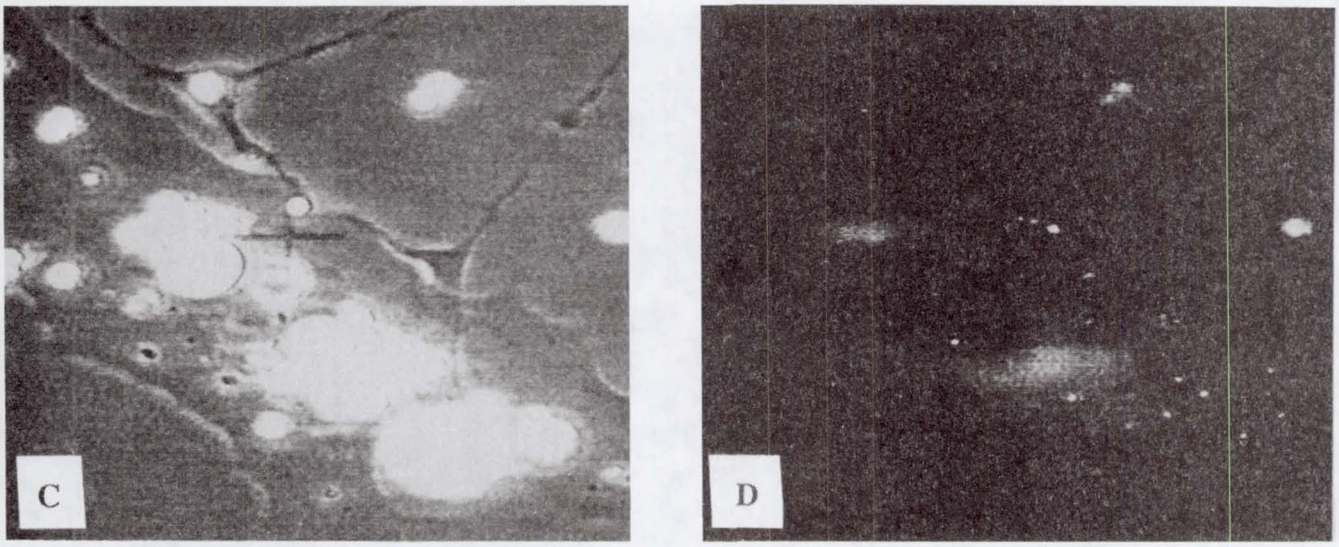
Our results suggest that during development a window of sensitivity exists during which exposure to simulated microgravity results in the compromise of an essential process in synapse development. These findings must be verified in actual microgravity before any extrapolations can be made concerning the contributions of gravity to synaptogenesis. They do, however, imply that embryonic development, which is known to be exquisitely sensitive to environmental perturbations, must be considered carefully in anticipation of the colonization of space by animals and humans.

EFFECTS OF VECTOR-FREE GRAVITY ON NERVE-INDUCED ACETYLCHOLINE RECEPTOR AGGREGATION

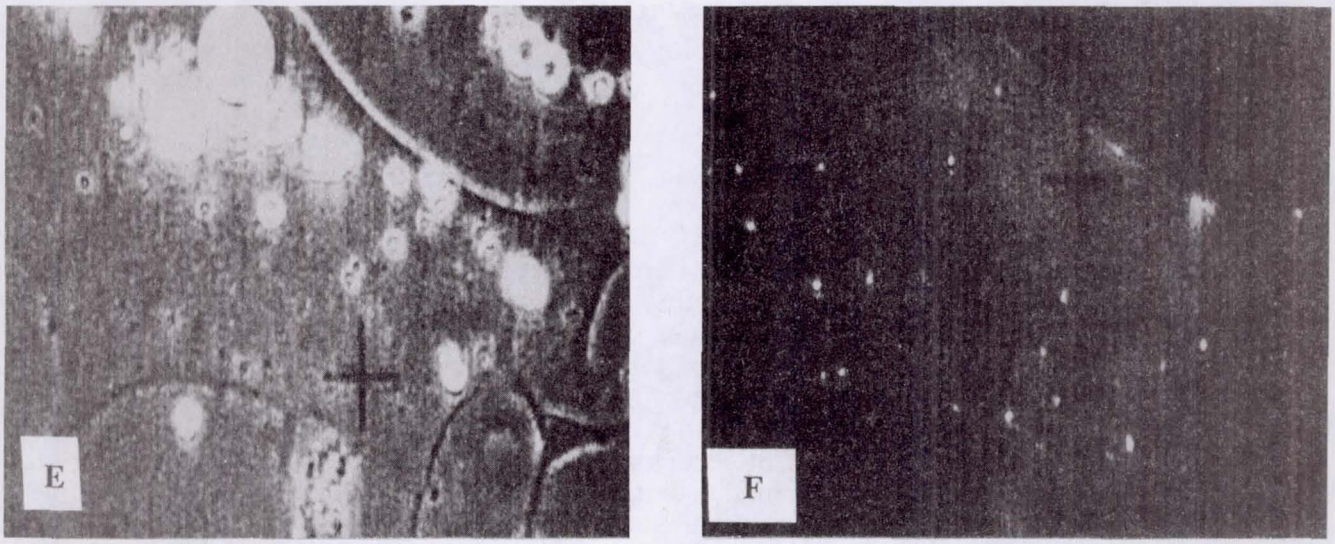
Figures A-F. Nerve and muscle cocultures were rotated for 24 hours in a horizontal clinostat at 1 and 10 rpm. Acetylcholine receptor patches were visualized with fluorescent rhodamine bungarotoxin.



Figures A and B. Control innervated myocyte (Figure A using phase-contrast microscopy) and its companion fluorescence image showing coincidence of brightly labeled acetylcholine receptors along the path of the neurite on the myocyte.



Figures C and D. Innervated myocyte from a culture rotated at 1 rpm showing significant reduction in fluorescent patching coincident with the path of the neurites.



Figures E and F. Innervated myocyte from a culture rotated at 10 rpm showing absence of fluorescence coincident with the path of the neurite.

THE EFFECTS OF GRAVITATIONAL FIELDS ON NEURAL SIGNALING IN THE HIPPOCAMPUS

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Description of Research

Our research has as a general long-term objective the elucidation of the effects of altered gravitational fields on neural regulatory mechanisms. Recently our experiments have dealt with the effects of the neurotransmitter serotonin in the hippocampus. We have selected the hippocampus for study because rats flown on Spacelab-3 showed an *increased* number of serotonergic receptors in this region of the central nervous system after 7 days in space. A more recent study has shown that rats exposed to a hypergravic field of 2.0 g for 7 days show a *decreased* number of receptors. Serotonin is a well-studied neuromodulator involved in many types of neural activities, including sleep and temperature regulation. In addition, in the hippocampus serotonin may modulate long-term potentiation, and hence may be associated with memory and learning. We wish to determine how serotonergic neural mechanisms are modified by altered gravitational fields.

On exposure to a hypergravic field or the microgravity environment of space temperature regulation is transiently impaired in rodents. This year we completed control studies covering the effect of temperature on several hippocampal mechanisms, including the effect of temperature on serotonergic modulation. In experiments conducted at Earth gravity (1 g), we found that diverse hippocampal neural mechanisms remained operational even though tissue temperature falls several degrees. Having completed these control experiments at 1 g, we have begun a study that will provide information on the modulation of electrical signaling in the hippocampus by serotonin before and after exposure to hypergravic fields.

Accomplishments

(1) This past year we have completed an initial study on the modulatory effect of serotonin on hippocampal neural activity. Figure 1 is a summary of this study. We recorded evoked activity over a wide range of tissue temperatures in the hamster hippocampal slice following repetitive Schaffer collateral stimulation. Population spike amplitude (the synchronous firing of hippocampal pyramidal cells) was measured after 10 μM serotonin (5-HT) was added to and then withdrawn from the perfusing medium. The temperature of the bath was fixed at different temperatures between 35°C and 15°C. Throughout this temperature range a depression in population spike amplitude of at least 10% was seen in 36 of 43 trials, with an average depression of 68%. No significant temperature dependence of the depressive effect was seen.

(2) Following perfusion with serotonin, the spike amplitude was enhanced at all temperatures, averaging 33% higher than control values (Figure 1). The rebound was strongest at 35°C and 15°C and weakest at 25°C.

(3) Long-term potentiation, seen as an enhancement of the amplitude of population spikes, was clearly evoked above 25°C by pulse train stimulation.

MODULATION OF NEURAL ACTIVITY IN THE HIPPOCAMPUS BY THE NEUROTRANSMITTER SEROTONIN

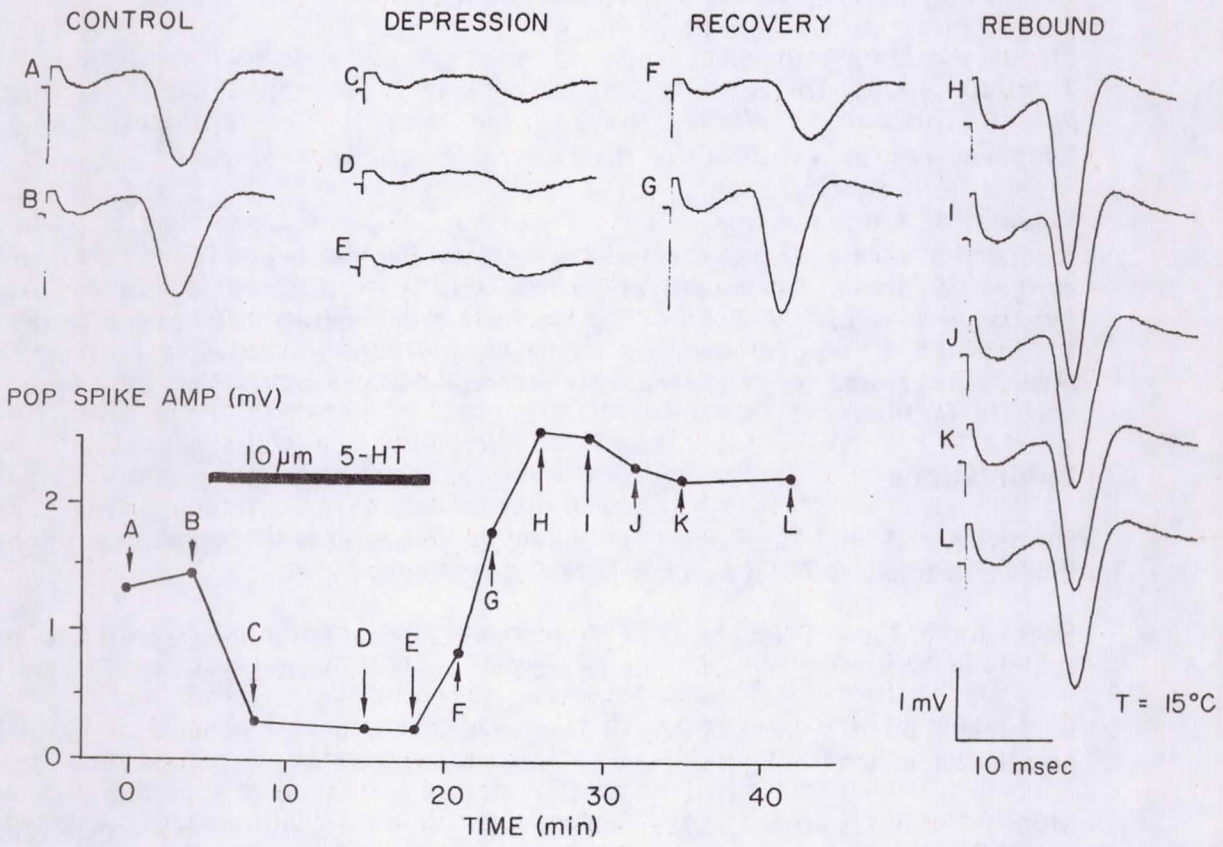

Figure 1. Repetitive Schaffer collateral stimuation of hamster hippocampal tissue over a wide range of temperatures was used to record neural activity. Population spike amplitude (mV) was measured after 10 µm serotonin (5-HT) was added to and then withdrawn from the perfusing medium.

(4) Experiments were completed on the neural regulation of temperature in hypergravic fields. Core temperature was found to be regulated in rats exposed to hypergravic fields, albeit at a lower level. Thus, even though the thermoregulatory system is impaired in hypergravic fields, and transiently falls several degrees centigrade, the rat then activates thermoregulatory mechanisms to control core temperature.

Significance of the Accomplishments

Finding #1: Depression of the amplitude of population spikes during serotonin perfusion was observed over a very large range of temperatures, from 35°C and 15°C. ***Throughout this temperature range a depression in population spike amplitude was seen showing that serotonin exerts this modulatory effect down to temperatures as low as 15°C.***

Finding #2: Following perfusion with serotonin, the amplitude of population spikes was enhanced over a very large range of temperatures. ***Thus, over the entire temperature range from 35°C to 15°C, serotonin was also demonstrated to exert an excitatory effect following the initial inhibition.***

Finding #3: Long-term potentiation is a form of synaptic plasticity that is widely studied as a cellular model for learning and memory in the vertebrate brain. ***Long-term potentiation can be elicited over a wide range of temperatures, and only at temperatures below 20°C is this neural mechanism blocked.***

Finding #4: On initial exposure to a 2 g or 3 g field, core temperature falls and then is regulated at a new set-point temperature several degrees below 37°C. ***Thus over the range of brain temperatures which are encountered upon exposure to hypergravic fields of 2 g to 3 g, neural mechanisms (discussed in Findings #1 through #3 above) are not adversely affected by changes in temperature. Over this range of temperatures serotonergic modulation of hippocampal activity is thus not impaired.***

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MECHANISM OF CONTROL OF BONE GROWTH BY PROSTAGLANDIN

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Description of Research

Biomedical studies of manned spaceflight have consistently shown a loss of weight-bearing skeletal bone. Various lines of evidence from humans and animals suggest that this loss is due to the lack of bone formation in the absence of gravity. The mechanism of control of bone growth is not known; however, clinical observations have demonstrated that increases of endogenous cortisol (2-5 fold over normal levels) as seen in Cushings syndrome is associated with bone loss and osteoporosis. Treatment of patients with asthma and rheumatoid arthritis has also demonstrated the deleterious effects of glucocorticoids on bone formation. The bone loss associated with glucocorticoids involves trabecular bone, and examination of patients treated with synthetic glucocorticoid prednisone shows a reduction in bone formation which was probably due to a direct inhibition of osteoblastic function.

These data suggest that the glucocorticoids could also play a role in the loss of bone that occurs in spaceflight. In the Skylab missions urinary corticols of 9 astronauts increased from an average of 54 ± 4 μg/total volume preflight to 94 ± 5 μg/total volume inflight. In individual crewmembers, urinary cortisols increased from 1.2 to 2.8 fold during flight. The direct cause of glucocorticoid-induced bone loss is not known. An understanding of the relationship between glucocorticoids and prostaglandins in bone cell growth and differentiation may increase our ability to understand the underlying mechanisms of bone formation.

The objective of this research is to understand the mechanism of bone loss in microgravity. Glucocorticoids and prostaglandins may play a role in the physiological control of bone cell growth and mineralization at the cellular and molecular level. We are using a cloned cell line, the MC3T3 osteoblast, to determine if: a) glucocorticoids affect prostaglandin synthesis and cell growth; b) if glucocorticoids affect bone mineralization; and c) if these glucocorticoid-induced changes are related to changes in protein synthesis.

Accomplishments

In these studies we have used 100 nM dexamethasone to simulate the increased plasma cortisols seen in spaceflight. We have established new methods of analysis for studying changes in the collagen synthesis in osteoblast cells.

(1) We have successfully established methods of SDSPAGE and western analysis of collagen type I in mineralized osteoblasts. By using affinity-purified antibody to type I collagen we have shown that MC3T3 cloned osteoblast makes type I collagen.

(2) ***Glucocorticoids decrease prostaglandin synthesis and decrease protein synthesis and DNA synthesis in growing osteoblasts.*** We have also established that the ***glucocorticoids inhibit prostaglandin synthesis and decrease the number of proliferating bone osteoblasts.***

(3) In the growing osteoblast, for every million cells, ^{14}C proline protein synthesis was decreased from 2040 $\pm$ 170 dpm/10^6 for control cells to 569 $\pm$ 36 dpm/10^6 for glucocorticoid treated cells. Total protein synthesis in the same experiment was decreased from 503 $\pm$ 28 μg to 316 $\pm$ 8 μg. Collagen synthesis was low in these non-confluent osteoblasts.

(4) ***In mineralizing osteoblast cultures, glucocorticoids cause a drastic decrease in ^{14}C proline protein synthesis*** over the 6-day period studied. Using western blots and autoradiography, we found that there was a decrease in the synthesis of both procollagen and collagen type I in the glucocorticoid-treated cells during this period.

Significance of the Accomplishments

Finding #1 establishes that the cloned osteoblast cell specifically synthesizes collagen type I and therefore adds another osteoblast-like characteristic to the MC3T3 line.

Findings #2 and #3 show that concentrations of glucocorticoids approximating the elevated concentration of cortisol seen in spaceflight inhibit osteoblast cell growth. These findings help to establish a relationship between glucocorticoid-induced inhibition of cell growth and inhibition of prostaglandin synthesis.

Finding #3 also establishes that glucocorticoids decrease protein synthesis, especially in proline-rich proteins.

Formation and mineralization of bone requires a collagen matrix formation. Finding #4 shows that slightly elevated levels of glucocorticoids can influence the formation of the collagen matrix by decreasing the synthesis of procollagen and its post processing to collagen. This, along with the other findings, suggests that the glucocorticoids may inhibit bone formation, both by interfering with the growth of the osteocyte, as well as by inhibiting synthesis of the collagen matrix.

Publications

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MICROGRAVITY-INDUCED EFFECTS ON PITUITARY GROWTH HORMONE CELL FUNCTION

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Description of Research

One of the major hormones made and released from the anterior pituitary gland is growth hormone (GH). This hormone is probably misnamed because it does many things in addition to being required for long bone growth. For example, it regulates metabolism of fat and carbohydrate and also controls proper functioning of the immune system. There are also indications that it participates in the complicated processes involved in wound healing, kidney cell function, and muscle metabolism. Since many of these GH target systems are affected by spaceflight, it is important to determine the effects of spaceflight on GH release from the pituitary.

Our laboratory has participated in three spaceflight experiments that address the issue of how microgravity affects GH release. In two experiments (Spacelab-3; Cosmos 1887), pituitary GH cells were prepared from rats flown 7-14 days. In another experiment (STS-8), dispersed pituitary cells were flown middeck locker at 37°C in closed tubes containing culture medium. On return to Earth, the GH-secreting capacity of the flight cells was compared with that from ground-based controls. The results from each of these experiments has shown that:

- *In vitro* release of GH is reduced by ~ 50% after flight
- *In vivo* release of GH is also reduced by the same amount (transplantation approach).
- The biological, but not necessarily the immunological, activity of the hormone is most affected by flight.
- Flight may preferentially affect release of high molecular weight forms of the hormone.

The issue of whether or not exposure of cells directly to the microgravity environment can affect cell function is one of current debate. Our STS-8 result, while preliminary, nevertheless offers important positive evidence for this concept. Indeed, it becomes stronger when coupled with data obtained from flight rats. There is an urgent need to "refly" pituitary GH cells under conditions where replication of cell culture vials can confirm the preliminary results of STS-8. In addition, experimental approaches to probe the nature of the cellular secretory defects are required. The goals of our flight experiment are therefore: (a) to establish and validate a closed vial cell culture system suitable for flight, and (b) to develop morphologic and functional tests that probe the mechanism(s) underlying the secretory defect.

It is obvious that an active ground-based research program is the best way to ensure success of a flight experiment. To this end, the long-term goals of our ground-based GH research program relate to an understanding of the molecular character of bioactive GH; identification of the mechanisms underlying processing of the GH molecule inside the GH

cell; and finally identification and localization of the GH cell subtype which produces bioactive GH.

Accomplishments

(1) A closed vial cell culture system (holding 165 vials) has been developed and found to be suitable for maintenance of primary rat pituitary cells for 9 days at 37°C. The secretory output of GH from cells in the vial compares favorably with that from cells cultured under usual laboratory conditions.

(2) A mRNA processing system for rat GH was characterized.

(3) A post-translational processing system for rat GH was characterized.

(4) A cell culture study of pituitary GH cells prepared from rats flown on Cosmos 1887 was completed (see Figure 1).

(5) A subpopulation of GH cells was isolated and shown to release large quantities of bioactive GH.

(6) The location of GH cells within the pituitary of individual rats has been defined.

(Many of these accomplishments have been achieved in collaboration with Dr. Richard Grindeland, NASA Ames Research Center.)

Significance of the Accomplishments

Finding #1 demonstrates the feasibility of doing a pituitary cell culture experiment in microgravity.

Finding #2 documents our ability to test for the presence of an alternatively spliced form of GH (20 kD) in culture media of flight cells.

Finding #3 enables us to probe for the molecular form(s) of the bioactive GH.

Finding #4 confirmed and extended the results of the Spacelab 3 mission (1985) which showed that ***the release of bioactive GH from pituitary cells of flight rats was compromised.***

Finding #5 demonstrates that ***not all GH cells produce a hormone with equal biological potency.***

Finding #6 demonstrates, for the first time, the ***asymmetric distribution of GH cells within the gland of the individual rat.***

Since GH controls many of the organ systems which are negatively affected by spaceflight, and since GH release from pituitary cells of rats is negatively affected by spaceflight, it is important to define the character of the "secretory lesion" in both molecular and cellular terms. In this context our future flight experiment seems particularly important, since it will establish if the secretory defect happens when cells themselves are directly exposed to microgravity.

MICROGRAVITY CELL CULTURE STUDY OF PITUITARY GROWTH HORMONE CELLS

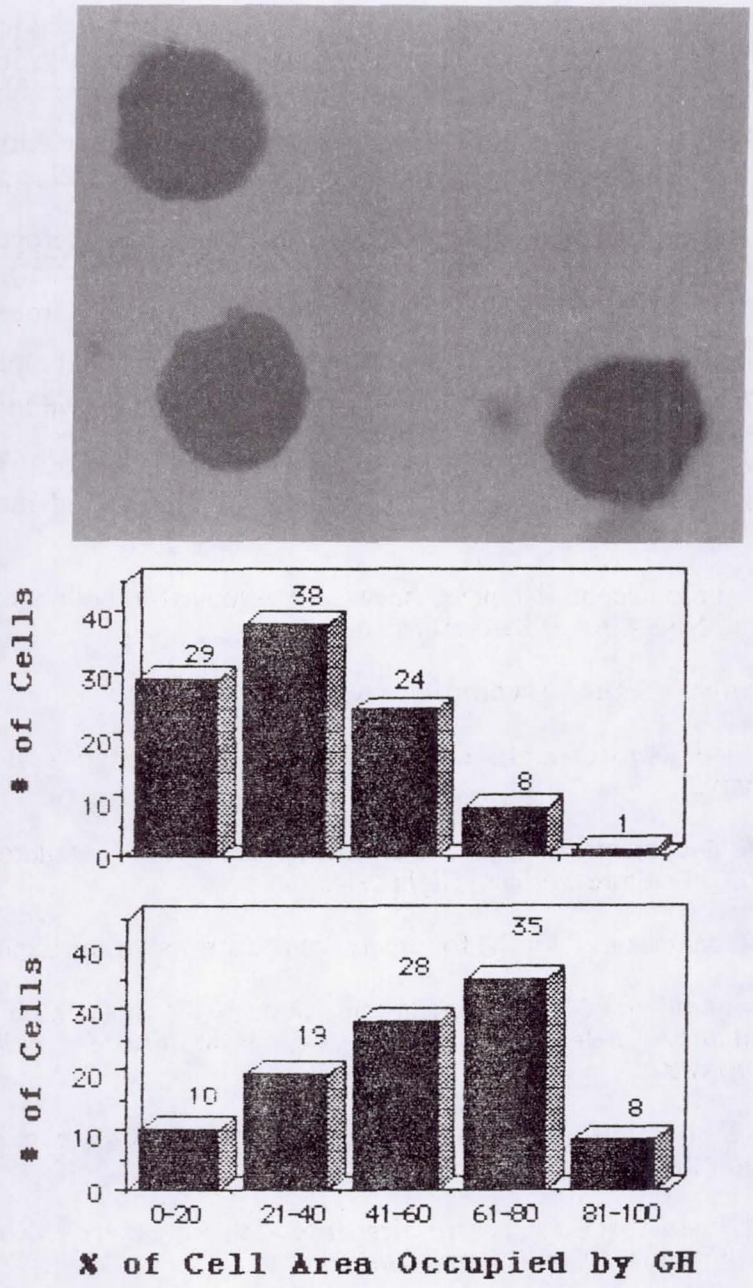

Figure 1. (Top) GH cells prepared from pituitary glands of rats flown on Cosmos 1887. These cells were immunochemically stained for intracellular, GH, and then subjected to image analysis. The percentage of the cytoplasmic area occupied by GH in cells prepared from the ground-based controls (middle) or flight animals (bottom) indicates a more diffuse distribution of hormone in cells of the experimental group.

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MECHANOCHEMICAL TRANSDUCTION ACROSS THE CELL SURFACE

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Description of Research

The long-range goal of this project is to characterize the mechanism by which mechanical signals are transmitted across the cell surface and transduced into alterations of intracellular biochemistry. Our working hypothesis is that physical forces may convey regulatory information through modulation of cell shape and associated alterations of cytoskeletal organization. This is based upon the observation that cells may utilize a "tensegrity" (tensional integrity) system of organization; cell shape results from the action of tensile forces generated by cytoskeletal microfilaments and resisted both by extracellular matrix (ECM) attachment points and intracellular microtubules. Recently, cell surface receptors have been identified that span the membrane and physically interconnect ECM molecules with specific intracellular cytoskeletal elements. Thus, as spreading cells form increasing numbers of interconnections between cell surface receptors and ECM molecules, they experience an increase in the magnitude of force that is exerted on their cytoskeleton. We propose that these mechanical forces may be transduced into biochemical information based upon changes of local thermodynamic parameters that alter cytoskeletal filament assembly; the function of many molecules involved in regulation of growth and metabolism depend upon the state of cytoskeletal integrity. This mechanochemical transduction system may also mediate gravity sensation in anchorage-dependent cells.

Our specific research aims include: (1) development of a defined *in vitro* system that can be used to study the effects of applying external mechanical loads to ECM receptors on the surfaces of suspended cells and (2) development of an *in vitro* system using attached cells to analyze the process by which transduction of cell-generated forces across the same transmembrane receptors produce cytoskeletal changes and modulate cell metabolism.

We have started to develop a controllable direct-current electromagnet system that can be used in conjunction with microparticles to apply mechanical tension to the surfaces of suspended endothelial cells. Our plan is to compare the effects of microparticles coated with non-specific ligands versus coatings with ECM molecules that are known to interconnect with cytoskeletal assemblies, such as fibronectin (FN). Simultaneously, we have begun to characterize the mechanism by which mechanical interactions between cells and FN modulate cytoskeletal organization and metabolism within cells attached to standard planar substrata. In addition, we have begun to explore an alternative method for applying external mechanical loads to attached cells (again via specific cell surface receptors) using a commercially available "Flexcell" system that utilizes elastic substrata. We believe that combination of these three different methods will allow us to begin to identify molecular mediators of mechanochemical transduction and to analyze the effects of external mechanical perturbation on cytoskeletal organization inside the cell.

Accomplishments

(1) ***Development of a Controllable Direct-Current Electromagnet:*** We are currently using a direct-current electromagnet (controllable from 0 to 100 amperes)

which, when energized to the maximum, produces a central field of approximately 7500 gauss at 0.5 cm from its pole tip without any associated electric field.

(2) ***Interactions Between Cells and Magnetic Microspheres:*** We have identified magnetic microspheres (4.5 μm diameter) that are perfectly spherical in form and extremely consistent in size. Application of a magnetic field results in magnetic microsphere alignment in a reversible, force-dependent fashion. More recently, we have developed a simple method for conjugating FN and bovine serum albumin (BSA) to these magnetic microbeads as well as a rapid quantitative assay for cell adhesion to these particles. ***Our results demonstrate that the number of cells that bound to fibronectin-coated beads increased in a dose-dependent fashion as the bead:cell ratio was increased. Importantly, under the same conditions, cells did not bind to beads coated with SBA, a non-specific ligand.***

(3) ***Studies with Attached Cells:*** Our greatest advances have been made in studies with attached cells. We have developed a chemically-defined, serum-free medium that will be used in all of our studies. Using this medium, ***we have been able to show that FN modulates cell growth by: (1) binding to cell surface integrin receptors, (2) promoting cell spreading, and (3) facilitating nuclear extension within cells attached to rigid, planar substrata. In constrast, suspended cells can bind soluble FN and small (4.5 μm) FN-coated microbeads, yet they do not change shape or grow if attachment to a rigid planar substratum is prevented.*** Results from additional studies suggest that FN-dependent alterations of cell shape exert their growth-modulating actions by both inducing microfilament assembly and modulating a central chemical signalling pathway, intracellular pH. Finally, we have been able to demonstrate that capillary endothelial cells can be switched between growth, differentiation, and involution modes by varying the mechanical integrity of their ECM adhesive sites.

(4) ***Development of an Alternative Method for Applying Physical Forces to Cell Surface Receptors:*** We have recently been allowed access to a commercially available "Flexcell Apparatus" which utilizes elastic substrata and controlled vacuum to apply mechanical loads to attached cells. Preliminary studies suggest that rhythmic extension (3 cycles per minute) of cells can stimulate their growth, and transduction of this regulatory signal depends upon the number of FN attachment points.

Significance of the Accomplishments

Finding #1: We plan to use the electromagnet system to focus on the effects of mechanical forces on early events during mechanochemical transduction, specifically changes in cytoskeletal organization and alterations of chemical signalling pathways.

Finding #2: The regularity of the size and shape of the microspheres that we are using should facilitate mathematical calculations of the effects of magnetic forces on cells as well as particle-particle interactions in future studies. Furthermore, we have now demonstrated that FN can be effectively conjugated to these magnetic particles with complete retention of its cell binding properties. Importantly, cell binding to coated particles is a highly specific process and this should facilitate analysis of specific paths of mechanochemical transduction across the cell surface.

Finding #3: Results of studies with attached cells clearly confirm that the growth and differentiation-modulating effects of ECM components depend upon their ability to provide a physical anchoring substratum that can resist cell-generated tensile forces and support cell extension. In other words, ***FN appears to convey regulatory information to the***

cell by: (1) binding to specific transmembrane integrin receptors, (2) resisting mechanical loads applied to those receptors, and (3) inducing changes in cytoskeletal organization. These findings add great support to our hypothesis that interactions between ECM molecules and their cell surface receptors may serve to alter cell function by transducing externally applied mechanical loads (e.g., gravitational forces) into changes of cytoskeletal structure.

Finding #4: This cell-stretch system will allow us to begin studies analyzing transduction of mechanical forces across integrin receptors while the magnetic system is being refined. However, it is important to point out that while stretching attached cells will facilitate analysis of the effects of physical forces on cells, this method will not permit us to discriminate between the effects of mechanical tension per se versus the effects of associated changes of cell shape or altered contact with the planar substratum. Nevertheless, use of this model in conjunction with magnetic perturbation of suspended cells should allow us to begin to address all of these important questions.

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SURFACE RECORDINGS OF VESTIBULAR RESPONSES TO PULSED ACCELERATION

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Description of Research

The principal aim of this research is to examine the role played by gravity in controlling or influencing the ontogeny of peripheral and central vestibular function. Ultimately, an in-depth comparison will be made of how vestibular function develops and matures under the influence of gravitational fields having strengths less than (< 1.0 g, hypodynamic), equal to (1.0 g), and more than (> 1.0 g, hyperdynamic) the natural gravitational field strength of Earth. Studies will be undertaken to examine how the gravitational vector strength and direction may modulate or influence normal responses to transient stimuli. Efforts will also be directed to investigate how vestibular function may adapt to changes in gravitational fields and to evaluate the vestibular systems' ability to adapt as a function of the organisms' age. Through these kinds of studies we may begin to appreciate the limits to, and determinants of, physiological adaptation in the vestibular system under a variety of gravitational environments.

The first year of research was undertaken to develop and evaluate a direct and noninvasive electrophysiological method capable of measuring the collective activity of afferent neurons in the vestibular system. The approach chosen was analogous to procedures commonly used to record peripheral and brainstem auditory neurons noninvasively. In the latter case, transient sound stimuli (e.g., clicks) are used to collectively activate auditory neurons. The collective activity of the auditory nerve (i.e., compound action potential) can then be detected and studied from the surface of the skull. A method was developed to deliver precisely defined pulsed linear acceleration stimuli to the cranium. Systematic variation of stimulus amplitude and rise time were key design features of the system.

Short latency vestibular responses to pulsed linear cranial acceleration were described for the first time (Jones 1987, Jones and Pedersen 1988). Responses were recorded noninvasively, consisted of four to seven dominant peaks and occurred within the first 8 msec following stimulus onset. Mean thresholds for responses were determined (0.12 ± 0.045 g). Response peaks did not invert upon stimulus inversion, were present in response to cranial but not trunk acceleration, were not attenuated by intense (98.5 dBSPL) broad-band auditory masking nor affected by ambient light conditions and disappeared with complete destruction of the labyrinth. The research demonstrated that responses were not dependent upon the auditory, somatosensory or visual modalities but were dependent on the activity of vestibular neurons bilaterally.

Current research seeks to: (a) complete explicit tests of the vestibular origin of responses, (b) examine whether or not similar responses could be recorded in mammals (rats), and (c) determine the best or adequate stimulus for response and evaluate the effects of altering stimulus rates.

Accomplishments

Explicit tests of the vestibular origin of responses: Complete bilateral destruction of the labyrinth by aspiration eliminated responses demonstrating that responses relied exclusively on VIIIth nerve sensory elements. High intensity (up to 100dB peSPL) white noise masking did not reduce responses, and bilateral removal of the cochleae (sparing the remaining labyrinth) did not abolish responses. These results demonstrate that the responses are vestibular.

Responses to pulsed linear acceleration in mammals: Responses to pulsed linear acceleration were studied in 16 rats (Lange 1988). A method of delivering transient linear acceleration stimuli was developed for rats. Stimuli were the same as used in birds. Mammalian response waveforms were similar to avian responses having four or more positive peaks occurring within 8 msec following the stimulus. The mean onset latency for P1 was 1.25 msec. Mean peak-to-peak response amplitudes ranged from .96 μv to 2.03 μv. The relationship between stimulus intensity and response latency was linear. Unlike the case in birds, in rats high intensity maskers reduced portions of the response. ***Response peaks resistant to auditory masking are considered to be candidates for vestibular components.*** Labyrinthectomies were not performed.

Effects of stimulus rise time: ***In both birds and mammals a significant relationship between stimulus rise time and response threshold was found.*** Threshold decreased in proportion to the rate of change in acceleration over time (da/dt). This suggests that the adequate or best stimulus may be the rate of change in acceleration (i.e., jerk) rather than the level of acceleration per se.

Significance of the Accomplishments

These studies set the stage for in-depth studies of the developing vestibular system in mammals and birds. These efforts lead directly to studies designed to evaluate the effects of hyper- and hypodynamic environments on the ontogeny of vestibular function. Responses to pulsed linear acceleration appear to depend on neurons that are more sensitive to the rate of change in acceleration than acceleration itself. Hypothetically, the responses therefore may reflect the activity of the so-called irregular type vestibular primary afferents.

Publications

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MOLECULAR BASIS OF TENSION DEVELOPMENT IN MUSCULAR ATROPHY

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Description of Research

The long-range goal of our research has been to assess the effects of immobility and hypokinesia on skeletal muscle function; in particular, the effect of exercise on skeletal muscle following prolonged atrophy. It is the aim of this project to describe the mechanism by which calcium ions and calcium-mediated physiologic mechanisms work in conjunction with gravity during hypokinesia.

Our research has focused on the atrophy-associated cellular changes which might predispose skeletal muscle to injury during reuse and recovery; specifically, the relationship between the length of sarcomeres, tension development and pCa (i.e., -log $[Ca^{2+}]$) in mechanically peeled fibers. Preliminary studies in this laboratory have shown that exercising atrophied skeletal muscle following hypokinesia produces myofibrillar damage and degeneration. A major finding was the appearance of Type IIC or transitional fibers and extensive fiber damage coincident with training during exercised recovery from hypodynamia. The damage appeared in the form of necrotic fibers, central nuclei, phagocytosis, and fiber debris in the intrafasicular and intrafibrillar spaces of the soleus.

Initial studies have sought to: (1) characterize the occurrence and distribution patterns of transitional and damaged fibers which occur in atrophied soleus during the course of recovery; and (2) verify the reliability and validity of the single skeletal muscle fiber histochemistry techniques as a method of classifying functional characteristics.

Accomplishments

(1) ***Spacial analysis of damaged skeletal muscle fibers following hypokinesia:*** Targetoid type muscle fibers have been described in association with the reloading of atrophied skeletal muscle during recovery from hypokinesia. These fibers, i.e., Targetoid or "moth-eaten" fibers, are characterized by a single pale staining central area of the affected fiber. Except for the cytological observation that the Targetoid fiber core has lost its ability to catalyze the splitting of ATPase acid and alkaline pre-incubations, the contractile and anatomical characteristics, as well as myosin isozyme composition of these Targetoid fibers in reloaded muscle are not known. Muscle fibers which are morphologically similar to these Targetoid fibers have been associated with disease and denervation. These fibers may be functionally impaired or nonfunctional. The occurrence and spatial distribution of these Targetoid fibers in cross-section during the 28-day course of a sedentary and a running recovery following 28 days of hypokinesia/hypodynamia unloading was measured.

When evident, the Targetoid fibers appeared in clusters of 2 to 8 fibers and seldom occurred singly. It has also been observed that Targetoid fibers (Figure 1) are consistently classified as Type I fibers in the soleus muscle using ATPase incubation at pH 10.3 and

4.3. Targetoid fibers were shown to occur maximally on day 14 of recovery following 28 days of hindlimb unloading in the rat. On day 14 of recovery, the maximum value of 3.8 $\pm$ 2.2 % ($\pm$ SEM) and 1.8 $\pm$ 1.8 % ($\pm$ SEM) was demonstrated for the running and sedentary groups, respectively. The sedentary group, though showing a lower percentage of Targetoid fibers, did show the initial occurrence at an earlier time during recovery, i.e., on day 7 of recovery the Targetoid percentage was 1.73 $\pm$ 0.80 % ($\pm$ SEM).

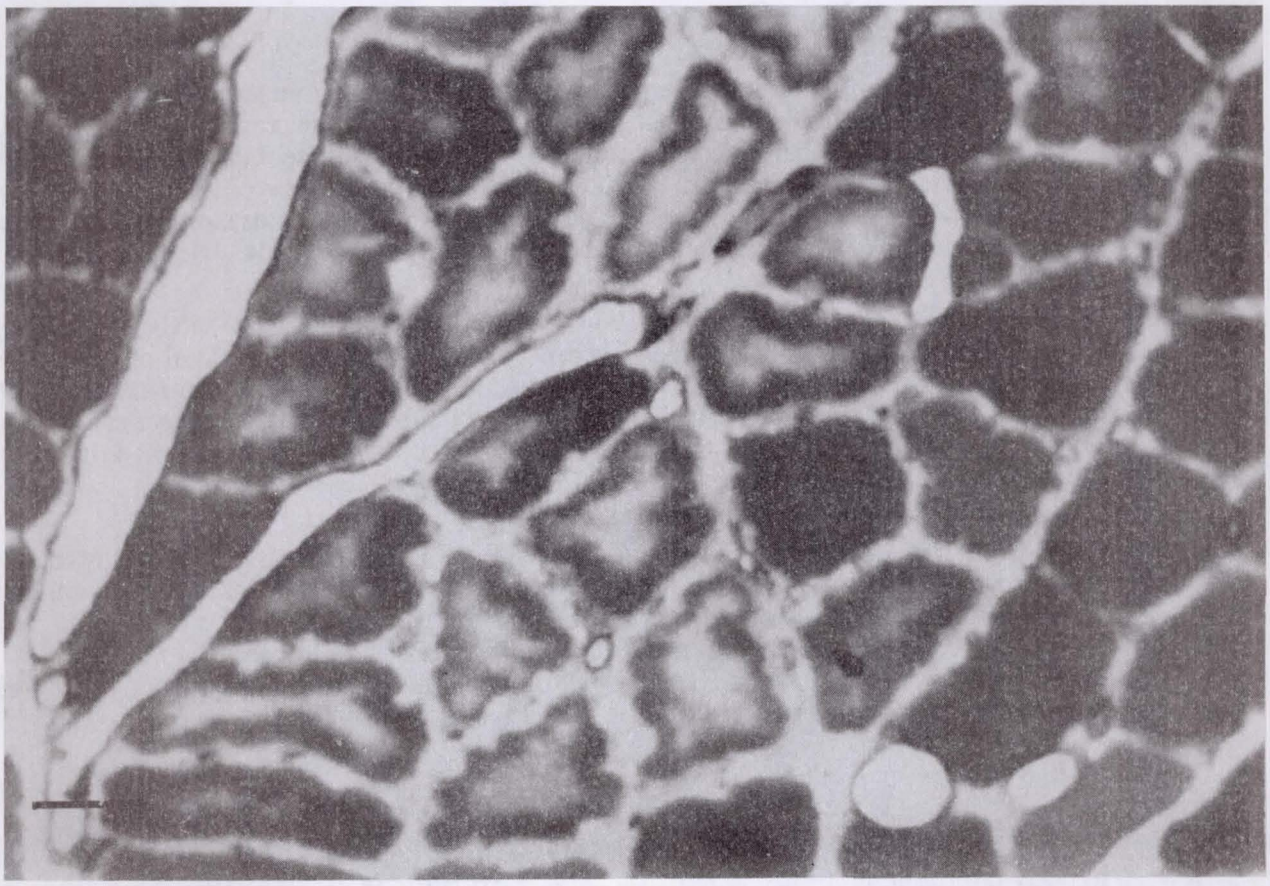

Figure 1. Serial section demonstrating clusters of "moth eaten" (Targetoid) central core fiber degeneration in soleus muscle from rats trained for 28 days following 28 days of hindlimb unloading. Myofibrillar ATPase, pH = 10.4. Bar = 100 μm.

(2) ***Histochemical identification of single atrophied skeletal muscle fibers:*** Previous investigators have found that single peeled (sarcolemma removed) skeletal muscle fibers which are histochemically identified from their myofibrillar ATPase and oxidative staining patterns correlate with functional properties. Our aim was to determine if single fiber histochemical techniques could be used to identify single peeled fibers from atrophied mammalian skeletal muscle. Adult female rats were subjected to 28 days of hindlimb unloading which resulted in decreases of 15% in mean body mass, 45% in mean soleus muscle mass (both significant at $p \leq 0.05$). Segments of single mechanically peeled fibers were divided and analysed for histochemical staining and the

remaining segment was used to analyse myosin isozyme composition. The maximum force-generating capacity of each fiber was examined using steady-state isometric force generation at saturating and subsaturating Ca^{2+} levels (pCa = 8, 5.4, 3.6; pH = (7.0; 22 $\pm$ 1°C), myosin isozyme composition (SDS-PAGE), and compared to fiber type from histochemical staining (myofibrillar ATPase; pH 10.4 and 4.6, and NADH tetrazolium reductase) in a total of 80 single mechanically peeled muscle fibers from atrophied and age-matched, non-suspended, control slow-twitch soleus.

Significance of the Accomplishments

Finding #1. The implications of this study are that hypodynamia structurally changes sub-cellular components of skeletal muscle which control functional capacity. The presence of these Targetoid type fibers in the reloaded soleus muscle of the recovering rat indicates a predisposition of the atrophied postural muscle to reuse injury. The fact that these abnormal fibers are found during a sedentary recovery indicates that simple weight bearing, if suddenly initiated, may be a sufficient load to cause injury to the atrophied muscle after only 28 days of hypokinesia.

The evidence that endurance capacity is diminished and postural skeletal muscle is at risk for structural damage following hypodynamia forms the foundation for further studies to determine specific structural effects that change with the intensity of exercise. As damage is caused by weight bearing alone following hypodynamia, a period of delay prior to remobilization should be evaluated.

Finding #2. These results demonstrate that the patterns of staining of the atrophied fiber segments are typical and distinguish type I from type II fibers. However, further subclassification of these fibers to types IIA and IIC was unreliable due to the marked increase in the relative amount of fast-type MHC's in these fibers. Single fiber histochemistry should not be used in atrophied muscle to classify fiber type without associated comparisons to cell matched SDS-PAGE electrophoresis.

Publications

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MECHANISMS OF VESTIBULAR ION TRANSPORT

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Description of Research

The specialized vestibular receptor cells, clustered into circumscribed "sensory epithelia," form part of a membranous fluid-filled compartment. The receptor cells are termed hair cells, owing to the numerous projections extending from their apical surfaces toward the fluid space. Deflection of these "hairs" by accelerational stimulation leads to increased passive flow of positively-charged ions from the fluid compartment, through mechanosensitive channels in the apical membrane, and into the negatively-polarized interior of the cell. This ionic "transduction current" exits at the basolateral membrane of the hair cell, toward the extracellular spaces within the sensory epithelium.

The fluid in the chamber opposite the hair cells (known as *endolymph)* is distinguished from all other extracellular fluids by its high K^+/low Na^+ ionic content. This property becomes more pronounced with phylogenetic advancement, so that endolymphatic $[K^+]$ attains its highest level in mammals (where the transduction current is carried almost entirely by potassium ions). The endolymphatic "ionic profile" is known to be maintained by active ion-transporting mechanisms, and the transduction current is consequently dependent upon a combination of mechanisms mediating both active and passive ionic flux. The long-term objective of this project is to characterize cellular mechanisms mediating active and passive ion transport in vestibular sensory organs.

There is reason to believe that acetylcholine receptors of the muscarinic type (MAChR) may modulate passive ion movements across the plasma membranes of vestibular hair cells and afferent sensory nerve fibers (see below). The goal of work carried out during the past year was to provide information on the distribution of MAChR in mammalian vestibular organs by autoradiographic grain-density analysis of presumptive muscarinic receptor sites.

Inner-ear sensory epithelia receive an innervation of central origin, distinct from the sensory afferent innervation. In mammalian vestibular organs, the centrifugal fibers terminate directly upon Type II hair cells, and upon the afferent nerve endings associated with Type I hair cells. Most lower vertebrates, however, have but one hair-cell type, upon which the centrifugal terminals make direct contact. Considerable evidence indicates that the centrifugal terminals utilize acetylcholine (Ach) as a neurotransmitter. In other systems, postsynaptic effects of Ach are mediated by two major classes of receptors — nicotinic and muscarinic — characterized by their respective responses to pharmacological agonists and antagonists. Earlier efforts to characterize inner-ear ACh receptors (AChR) focussed on the mammalian auditory system. Electrophysiological responses at the auditory periphery are influenced predominantly by nicotinic agents, and muscarinic AChR density is reportedly low within auditory sensory epithelium. Vestibular AChR have, until now, been studied almost exclusively in lower vertebrates, where electrophysiological responses to nicotinic agents are, once again, prominent. Recent electrophysiological studies, however, indicate that ACh receptors with muscarinic properties are present on hair cells of the frog ampulla and modulate afferent neural discharge. Moreover, recordings from isolated hair cells of

fish saccule indicate that muscarinic agents modulate an outwardly-directed potassium current at the basolateral membrane.

In order to identify possible muscarinic ACh receptors in mammalian vestibular organs, we isolated these tissues from the gerbil inner ear by microdissection. The specimens were then incubated *in vitro* with 1 nM [^{3}H] quinuclidinyl benzilate (^{3}H-QNB), a specific antagonist with high affinity for muscarinic ACh receptor sites. Control preparations were incubated in medium supplemented with 10 μM radioinert atropine (a second muscarinic antagonist) to block specific binding of ^{3}H-QNB, and thereby demonstrate possible nonspecific binding. Following incubation, tissues were processed for light-microscopic autoradiography. Autoradiographic grain densities (GD: grains/μm^2 tissue area) were determined by morphometric techniques, and evaluated by two-tailed t-tests. Background grain density was measured in the endolymphatic compartment opposite the respective sensory epithelia, and tissue grain densities were corrected for background.

Accomplishments

(1) We have improved the resolution of the autoradiographs by use of paired dark-field and phase-contrast micrographs. The former permit resolution of individual silver grains (i.e., QNB binding sites), even at the low magnifications necessary to produce survey micrographs of the macular organs (Figure 1a). The latter permit relatively good resolution of cellular detail (Figures 1b, 2a).

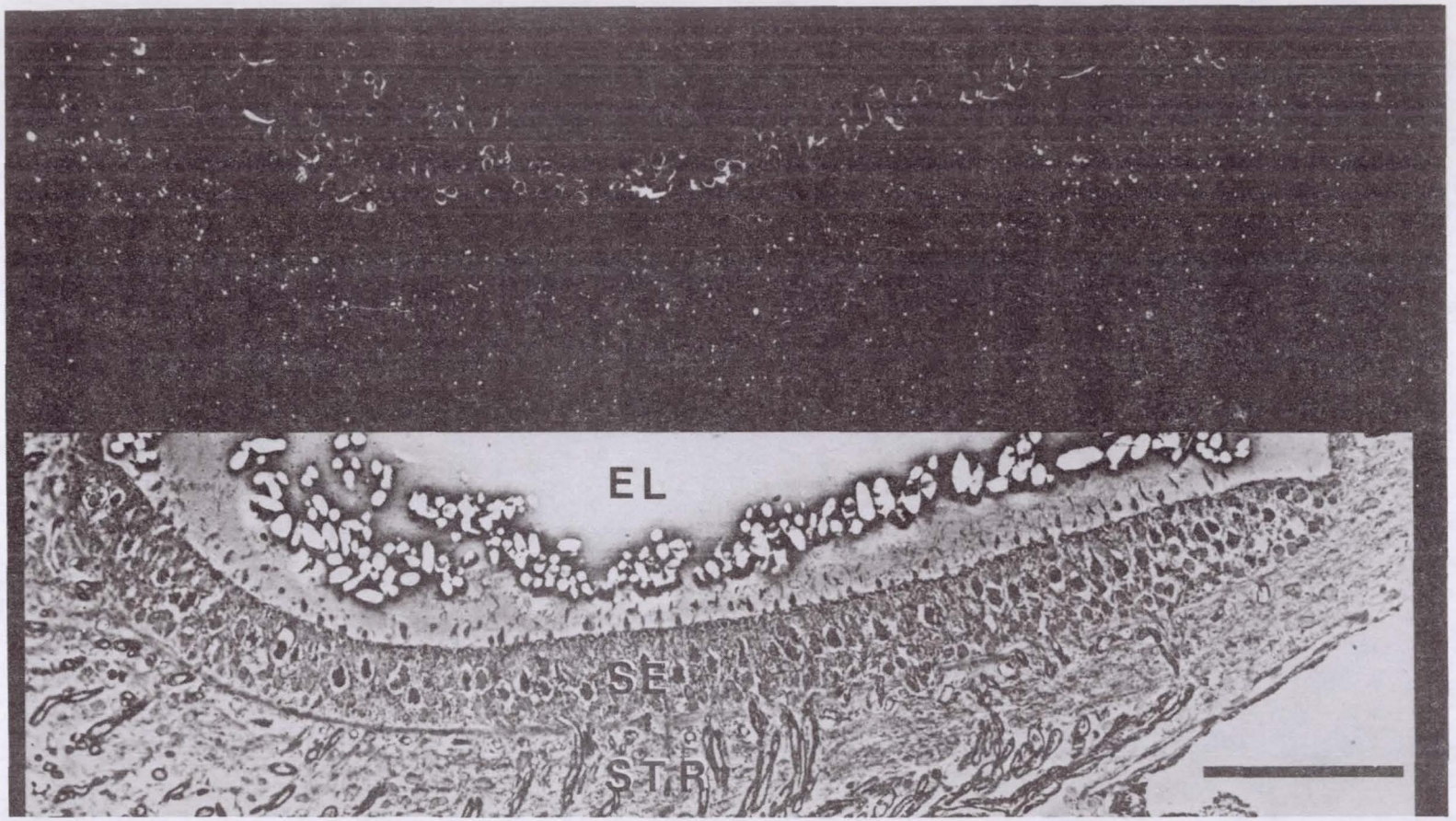
(2) Grain-density measurements of control preparations incubated with excess unlabelled atropine showed that no tissue region in the controls was labelled significantly above background. Moreover, label density of sensory epithelium in tissues incubated with ^{3}H-QNB alone was significantly higher than that of sensory epithelium from atropine controls ($p<10^{-7}$: cf. Table 1).

(3) Label density of sensory epithelium from experimental preparations of ampulla, utricle and saccule was significantly higher than (a) background ($p<10^{-4}$); or (b), that of adjacent stromal tissue (comprising connective tissue, nerve fibers, and capillaries: cf. Figure 1b) ($p<10^{-4}$: cf. Table 1).

(4) In utricle and ampullae incubated with ^{3}H-QNB only, we partitioned the sensory epithelium into three layers of comparable "depth." The "middle layer" (which encompassed both the synaptic poles of the hair cells and the centrifugal nerve terminals on hair cells and afferent nerve endings), showed significantly higher labelling ($p<.05$) than the "apical layer" (which encompassed the hair-bearing apical surface of the sensory cells).

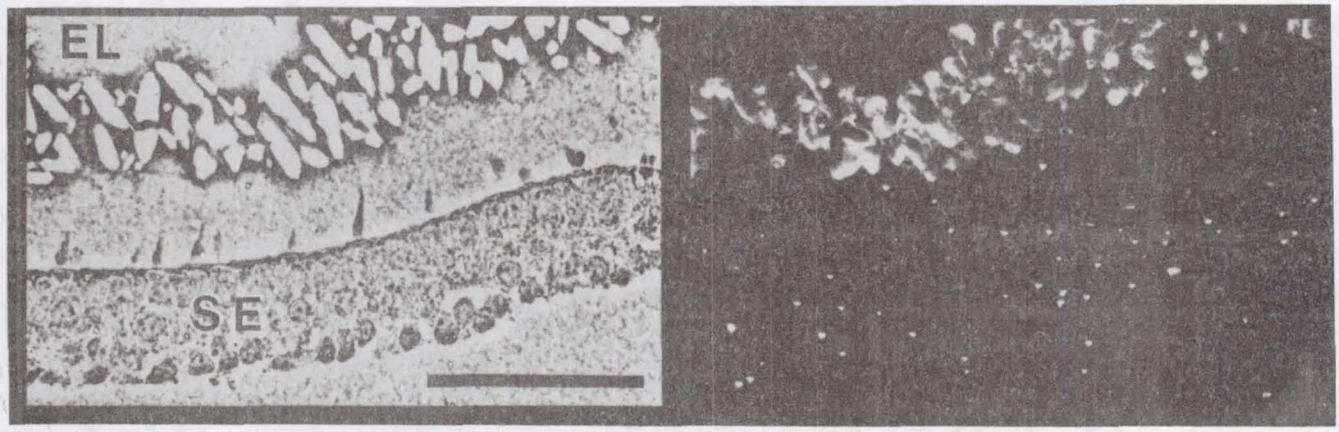
(5) Label density in sensory epithelium of ampullae incubated with ^{3}H-QNB was significantly higher than that of sensory epithelium from experimental preparations of utricle or saccule ($p<10^{-4}$: cf. Table 1).

(6) Label density over nerve bundles passing through the stromal tissue of ampullae incubated with ^{3}H-QNB was higher than either label density in nerve bundles from control ampullae incubated with QNB + atropine ($p<.05$) or label density in stromal tissue of ampullae incubated with QNB only ($p<.02$).



Figures 1a and 1b. This pair of micrographs illustrates the macula of the utricle following incubation with 1 nM ^{3}H-QNB and an autoradiographic exposure of 78 days. Figure 1b is a phase-contrast micrograph demonstrating the cellular detail of the utricle. SE: Sensory epithelium; STR: Stromal tissue; EL: Endolymphatic compartment. Figure 1a is a darkfield micrograph of the same section at identical magnification, illustrating the distribution of autoradiographic silver grains over the various utricular tissues. Note that the highest density of silver grains (which appear as white "dots") is found over the sensory epithelium. Grain density is lower over stromal tissue, and lowest of all over the endolymphatic compartment. Calibration (Figure 1b): 100 µm.

DISTRIBUTION OF QNB BINDING SITES IN VESTIBULAR MACULAE



2a 2b

Figures 2a and 2b. This pair of micrographs illustrates the detached sensory epithelium of the saccule following incubation with 1 nM ^{3}H-QNB and an autoradiographic exposure of 78 days. Figure 2a is a phase-contrast micrograph which demonstrates the cellular detail of the sensory epithelium (SE), while Figure 2b is a darkfield micrograph, at identical magnification, which highlights the distribution of autoradiographic silver grains. Grain density is much higher over the sensory epithelium as compared to the endolymphatic compartment. Calibration (Figure 2a): 50 μm.

TISSUE AND INCUBATION CONDITIONS	GRAIN DENSITY (Grains per $\mu m^2 \times 10^{-3}$, ± std. deviation)		
	Sensory Epithelium(1)		Stromal Tissue(1)
AMPULLA: Atropine (n=5)	0.4 ± 1.8	$p<10^{-7}$	-0.7 ± 1.6
AMPULLA: QNB (n=9)	11.2 ± 1.5		1.4 ± 3.1
UTRICLE: QNB (n=6)	6.8 ± 1.1	$p<10^{-4}$	2.3 ± 1.2
SACCULE: QNB (n=5)	8.4 ± 1.0	$p<10^{-4}$	1.7 ± 0.2

Table 1. *Grain-density measurements of isolated vestibular organs.* Isolated vestibular organs were incubated (2 hr, 25° C) with 1 nM ^{3}H-QNB ("QNB"), or with 1 nM ^{3}H-QNB + 10 μM unlabelled atropine ("Atropine"). Autoradiographs were processed in parallel, with an exposure of 78 days. Grain densities in sensory epithelia were significantly higher than those in stromal tissue ($p<10^{-4}$), excepting ampulla incubated with QNB + Atropine (not significant: $p>10^{-1}$).

(1)Corrected for autoradiographic background as described.

Significance of the Accomplishments

Finding #1: These improvements in methodology resolve individual receptor sites in survey micrographs depicting a number of different tissue regions within a particular vestibular organ. Quantitative estimates of receptor densities in heterogeneous vestibular tissues are now possible. These techniques should be valuable for the study of other putative neurotransmitter and neuromodulator receptor sites in the vestibular apparatus.

Findings #2, 3, and 4: These results strongly support the supposition that the observed QNB binding sites represent actual muscarinic ACh receptors. Thus, finding #2 indicates that ***binding of labelled QNB was significantly reduced by competition of unlabelled atropine*** (a chemically-distinct muscarinic antagonist) for specific binding sites. Findings #3 and #4 show that the distribution of vestibular QNB binding sites is consistent with the known distribution of centrifugal cholinergic nerve terminals.

Finding #3: ***The demonstration that presumptive MAChR are statistically most numerous within vestibular sensory epithelia stands in contrast to previous reports that muscarinic receptors are less numerous in auditory sensory epithelium than in non-sensory auditory structures.***

Finding #5: Since densities of presumptive MAChR are significantly higher in sensory epithelium of the ampullae than in sensory epithelia of the maculae, it is possible that muscarinic function may differ in the respective vestibular receptor organs.

Finding #6: In mammalian vestibular organs, afferent nerve endings on Type I hair cells are known to receive centrifugal cholinergic innervation. ***The demonstration of significant label density associated with vestibular nerve fibers therefore raises the possibility that these QNB binding sites represent muscarinic receptors "in transit" from afferent nerve cell bodies to afferent terminals on Type I hair cells.***

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COLLAGEN SYNTHESIS, ASSEMBLY, AND MINERALIZATION IN CHICKEN OSTEOBLAST CELL CULTURES

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Description of Research

The purpose of this research is to describe the basic molecular biology and biochemistry, morphology, and ultrastructure of a bone cell culture system to be used for inflight experiments under zero-gravity conditions. At the current time, we are conducting investigations in the laboratory under normal gravity. We are interested in defining the growth and development of cultured bone cells derived from chicken calvaria osteoblasts and in studying the reproducible onset and progression of mineralization in the culture extracellular matrix.

The cultured embryonic chick osteoblasts mineralize in a temporal sequence of differentiation of the osteoblast phenotype. Over a 30-day culture period, parameters of both cell and extracellular matrix development have been assayed, including determinations by biochemical or electron microscopic means of collagen gene expression; collagen synthesis, processing, and accumulation; cellular growth; presence and function of non-collagenous proteins; and extracellular matrix assembly and mineralization. Basic questions of interest concern (1) what are the mechanisms of collagen gene expression, (2) how are observed temporal increases in collagen cross-links related to the formation of collagen ultrastructure, (3) how are the collagen fibrils of the extracellular matrix assembled into orthogonal arrays, (4) what is the precise interaction between the collagen fibrils and mineral deposition in the cultures, and (5) what are the effects of non-collagenous proteins on mineralization in the system?

Accomplishments

The investigations defining the culture system have established thus far that:

(1) A reproducible timed sequence of proteins, including Type I collagen and non-collagenous proteins (alkaline phosphatase, osteocalcin, and a number of phosphoproteins), is developed in the cultures, and an extensive elaboration of an extracellular matrix is established which mineralizes beginning by day 6 of the 30-day culture period.

(2) Maximal collagen synthesis occurs in the cultures between days 3 and 12, followed by a 3-6 day lag before maximal collagen accumulation is found between days 12 and 24.

(3) The rates of procollagen processing are 6 times more rapid by 30 days of culture than at 3 days of culture.

(4) In work done in collaboration with Dr. David Eyre (University of Washington, Seattle), specific, non-reducible pyridinium cross-links unique to collagen and stabilizing individual synthesized molecules are found to appear in the cultures with extracellular matrix development.

(5) The formation of extracellular matrix by synthesized collagen fibrils results in a superfibrillar arrangement of mutually orthogonal fibril bundles.

(6) Alkaline phosphatase, osteocalcin, and a number of phosphoproteins are present in the culture system, appearing at specific times during the culture elaboration. One of the phosphoproteins (66kD) is found by immunocytochemistry to be localized within small foci containing calcium and possibly representing sites of initial mineral deposition in the extracellular matrix.

(7) Mineralization of the culture system occurs in close association with collagen. The nature of the mineral is a calcium phosphate, identified as poorly crystalline hydroxyapatite.

Significance of the Accomplishments

The importance of all the findings outlined above is that they correspond to respective observations made *in vivo* and published extensively. In addition, the result cited in Finding #2 above suggests that ***extracellular matrix formation is dependent on post-translational events affecting collagen accumulation and not collagen synthesis per se.*** The result in Finding #3 suggests further that ***collagen accumulation is not dependent on the rate of synthesis but may also be dependent on the efficiency of collagen fibril formation.*** Cross-linking and matrix formation results in Findings #4, #5, and #7 imply that collagen fibril diameters increase by lateral addition of smaller units to pre-existing fibrils, and that such additions and new fibril formation are concurrent. Cross-link formation is constant with time and generally correlated with the observed increases in collagen fibril diameter. From Finding #7, mineralization occurs only if a preliminary matrix is present in the system. Overall, the culture system exhibits biochemical and ultrastructural characteristics similar, if not identical, to those of embryonic bone *in vivo*. It provides an excellent and reproducible model by which cellular differentiation, the associated gene expression of bone proteins, and extracellular matrix mineralization may be studied under zero-gravity conditions of spaceflight following the current determination of basic parameters of the cultures grown under normal gravity.

Publications

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MICROMOTIONAL STUDIES OF UTRICULAR AND CANAL AFFERENTS

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Description of Research

The long-range goal of this research is to refine our understanding of the sensitivity of the vestibular components of the ear to very-low-amplitude motion and, especially, the role of gravity in this sensitivity.

To date we have focused on the American bullfrog, a common animal subject for vestibular sensory research. Our principal experimental method is to apply precise, sinusoidal microrotational stimuli to an anesthetized animal subject, to record the resulting responses in an individual vestibular nerve fiber, and to use intracellular dye to trace the fiber and thus identify the vestibular sensor that gave rise to it. In this way, we are able to identify specific micromotional sensitivities and to associate those sensitivities definitively with specific sensors. Furthermore, by recording from nerve fibers after they leave the intact inner-ear cavity, we are able to achieve these identifications without interrupting the delicate micromechanics of the inner ear. We are concerned with the relative roles of the otoconial organs (saccule and utricle) and the vertical semicircular canals in the sensing of microrotational motion of the head about horizontal axes, and with the role of gravity in mediating the sensitivity in the otoconial organs.

The functional characterization of individual nerve fibers is accomplished with a conventional analytical tool, the cycle histogram, in which the nerve impulse rate is plotted against the phase of the sinusoidal stimulus. Since nerve impulses are generated by a nonlinear process, there is distortion inherent in the translation of signals from continuous generator potential at the vestibular sensory transducer to the train of impulses in the nerve fiber. During the past year, in order to determine the distortion that the impulse generation process would introduce, we also carried out extensive theoretical and computer modeling studies of that process.

Accomplishments

Individual vestibular afferent axons in the American bullfrog were functionally characterized in terms of the amplitudes and phases of their impulse rate modulation in response to sinusoidal rotational stimuli. ***Using intracellular dye to trace these functionally characterized vestibular afferent axons to their peripheral origins, we definitively separated anterior vertical canal axons from utricular axons and showed that the traditional functional criteria are inadequate for that purpose;*** there is a sizable population of utricular striolar axons that mimic the function of anterior vertical canal axons sufficiently to be mistaken for the latter under those criteria. Refining the functional characterization, we found conspicuous distinctions between a subset of the utricular striolar axons and vertical canal axons; for other striolar axons the distinctions are quite subtle and perhaps unreliable. Interestingly, for small-amplitude, low-frequency rotations about horizontal axes, the impulse rate modulation in this distinctive subset of utricular striolar axons represents rotational velocity

more faithfully (in both phase and amplitude) than does that in any of the vertical canal axons we studied. ***The stimulus for this faithful representation of rotational velocity is the rate of change of the projection of the gravity vector onto the utricular surface.*** In most axons it extends to frequencies in the neighborhood of 0.1 Hz; in others it extends somewhat lower. The upper frequency limit is abrupt and occurs when kinetic jerk (which is proportional to the third power of frequency) becomes comparable to the rate of change of the projected gravity vector. That in turn depends upon the position of the axis of rotation relative to the ear. For rotations about the center of the head, in both frogs and humans, the upper limit would be between 2 and 3 Hz.

Our dye-tracing studies showed a strong correlation (but not a perfect one-to-one mapping) between the number of hair cells innervated by each vertical canal axon and the transducer gain of that axon. (The gains of dye-marked axons ranged from 1 to 30 impulses/second per degree/second, the number of hair cells innervated ranged from 7 to 30.) Transducer gain did not seem to correlate strongly with the locations or types of hair cells innervated. Dye tracing to the bullfrog utricle confirmed the functional organization reported by Baird and Lewis (1987), ***implying that the faithful rotational velocity sense arises from the (phasic) hair cells at the very center of the utricular striola.***

To determine the distortion that impulse generation must introduce into impulse-rate response waveforms, we undertook a thorough modeling study of impulse generation. We concluded that as long as adequate noise is present at the impulse-generation locus, the cycle histogram will faithfully represent the amplitude and phase of small-amplitude sinusoidal generator potentials. Furthermore, we identified the form of distortion that must be introduced by the impulse generator as the amplitude of a sinusoidal generator potential becomes large; we concluded that most of the distortion observed in our vestibular cycle histograms under those circumstances is a consequence of impulse generation, rather than nonlinearities in the vestibular transduction process. Occasionally, we found a form of distortion (shift in mean impulse rate) that could not be explained in terms of the impulse generator, and therefore must be attributed to the transducer. A similar form of distortion seems to accompany adaptation in inner-ear acoustic transducers.

Significance of the Accomplishments

It is well known that the human vestibulo-ocular reflex operates very well for head rotations that are a small fraction of a degree. Traditionally, the sensors responsible for this reflex have been assumed to be the semicircular canals. The otoconial organs of mammals have been considered sensors of lineal motion and of head orientation relative to gravity. Our observations of gravity-mediated rotational velocity sensitivity in the frog casts serious doubt on this traditional view. ***Furthermore, our results suggest that serious deficits in rotational motion sensitivity could occur in microgravity environments.***

As far as we know, our results are the first demonstration of correlation between the transducer gain of an inner ear sensory fiber and the number of receptor cells connected to that fiber.

For several decades, biophysicists and theoretical neurobiologists have had excellent descriptions of the properties of isolated impulse-generating sites from neurons, but they have been unable to reconcile those properties with the impulse coding that actually takes place in a large proportion of intact neurons. What we have demonstrated is that the missing ingredient was noise. ***We have shown that noise linearizes the impulse-initiation process and allows continuous signal modulation in neural***

networks. This sort of modulation is present in most nerve fibers carrying vestibular signals to the brain.

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GRAVITY AS A PROBE FOR UNDERSTANDING EMBRYONIC PATTERN SPECIFICATION IN THE AMPHIBIAN MODEL SYSTEM

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Description of Research

The development of the fertilized amphibian egg is utilized as a model system to analyze the effects of gravity on a single cell. The amphibian egg provides the following primary advantages for gravitational studies: (a) clear gravity responses such as a gravity-driven rotation upon fertilization; bilateral polarity can be determined experimentally by orientation of the fertilized egg with respect to gravity; (b) large size of egg; (c) readily tracked intracellular components; and (d) ease of experimental manipulation. The goal of this research is to understand the cellular and molecular basis for the way the amphibian egg as well as its intercellular components respond to novel gravitational situations such as hypergravity (centrifugation), hypogravity (horizontal clinostat rotation, spaceflight), and orientation of the egg with respect to the gravity vector (e.g., inversion). Emphasis is placed on two basic phenomena in developmental biology: (a) the manner in which the amphibian egg cytoplasm is organized and (b) the extent to which the reorganization of the cytoplasm drives embryonic pattern specification (bilateral symmetrization).

Accomplishments

(1) ***Identification of a new class of amphibian eggs that display a preprogrammed bilateral symmetry.*** Careful analysis of the polarization of amphibian eggs with respect to the sperm entrance site revealed that a subpopulation of eggs within a typical spawning appear to be preprogrammed. Their polarity is not determined by the sperm but by a built-in gravitational bias (tilt of the egg's animal/vegetal axis with respect to the gravity vector).

(2) ***Establishment of an expanded and more precise classification of the apparent cytoplasmic viscosity of inverted fertile amphibian egg cytoplasm.*** The degree of cytoplasmic movement in inverted fertile eggs can be quantitated as cytoplasmic mobility (CM). The number of batches of eggs analyzed and the resolution of the measurements was improved. The number of CM classifications was increased from three to five. The increased number of classes has allowed us to identify a wider range of variability between different spawnings of eggs.

(3) ***CM can be correlated with the egg's gravitational response.*** Under normal 1 g conditions eggs of all CMs appear to have an equal chance of developing normally. However, eggs subjected to altered force environments, such as inversion, respond differently depending on their intrinsic CM. There is a direct correlation between CM and inverted egg survival, inverted egg primordial germ cell numbers, and failure of inverted eggs to gastrulate properly.

(4) ***Variation in CM may involve microtubules.*** Conditions that depolymerize microtubules (cold shock) reduce the variation in CM and decrease the apparent cytoplasmic viscosity.

(5) ***The amount of polymeric tubulin within whole eggs does not correlate with the CM.*** The amount of polymeric beta tubulin levels in eggs from batches of fertile and unfertilized eggs or from batches of eggs with different CMs was analyzed. Different batches of eggs with extreme differences in CM can have similar polymeric beta tubulin levels.

Significance of the Accomplishments

Not all eggs are built identically during oogeneses and the variability of the response of fertile amphibian eggs to a given force environment can be substantial (Findings #1, #2, and #3). The variability in CMs and the reduction in that variability upon microtubule depolymerization (Finding #4) indicate that the variable response of amphibian eggs to gravitational perturbation (Finding #3) may well involve the amount and/or organization of the egg's cytoskeleton, especially microtubules. The observation that the polymerized tubulin levels of eggs did not correlate with the CM of the cytoplasm of whole eggs (Finding #5), in light of the cold shock data, implies that the localization and pattern of polymerized tubulin distribution may play an important role in CM. These accomplishments show that an understanding and appreciation of the basis for variability in the organization of the cytoplasm and cytoskeleton will be essential in properly interpreting data from gravity perturbation experiments, including spaceflight data.

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STRUCTURAL DEVELOPMENT AND GRAVITY

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Description of Research

The goal of this research is to understand the role of gravity in skeletal growth and development. To achieve this goal, we must first learn what turns bone cells on and off; if/how these cells communicate with each other and with their environment; if/how secretory products are altered by different gravity levels and how alterations in the organic matrix might affect mineralization and strength; and determine the role of local and systemic factors (including endocrine, blood flow, and fluid shifts) in these responses. To accomplish these studies, both spaceflight experiments and flight simulations are essential.

Gravity is a major factor determining the amount of structural support required by Earth organisms. The hypothesis of this research effort is that skeletal support structures will change during spaceflight and that the magnitude and duration of change will be dependent upon the modeling or remodeling activity in each bone and the length of exposure to spaceflight; changes in both quantity and quality of bone will occur. Most ground-based research is done in rats exposed to simulated spaceflight. Three flight experiments have been approved and will allow gathering of more information to support or negate the hypothesis.

Accomplishments

1) Analysis of ***Cosmos 1887 rat tissues showed major defects in tibial periosteal vascularity while maxillary progenitor populations appeared to recover during the 55 hr postflight period*** (collaborative studies with Dr. Steve Doty and Dr. Eugene Roberts).

2) Preliminary results from a null magnetic field study suggest that ***the soleus muscle is more atrophied in unloaded rats without a magnetic field,*** but no changes were noted in bone chemistry.

3) Preliminary ***observations in rat bone cell cultures suggest that bone cells grown on collagen-coated beads become phagocytic under certain culture conditions*** (collaborative study with Dr. Steve Doty).

4) Histomorphometry techniques were upgraded using a Mac II and modified NIH image analysis software.

Significance of the Accomplishments

Finding #1 suggests that microvascularity at the surface of the bone shaft is altered by spaceflight and might be associated with, or perhaps even initiate, the changes in bone maturation. The recovery of the progenitor bone cell population in the maxilla occurred rapidly in the very stressful postflight period following this 14 day flight, suggesting that readaptation to gravity overcomes the usual suppression of corticosteroids at this sampling site.

Finding #2 suggests that soleus muscle mass may require magnetic fields to prevent drastic atrophy when the muscle is unloaded. This experiment must be repeated to verify this unexpected result. Although no change was noted in bone chemistry, the histomorphometry measurements have not been completed.

Finding #3 suggests that under certain culture conditions, rat osteoblast-like cells grown on collagen coated beads become phagocytic and actually engulf part of the collagen bead rather than producing bone matrix. Thus, these cells are capable of both degradation and synthesis.

Publications

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SKELETAL COLLAGEN TURNOVER BY THE OSTEOBLAST

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Description of Research

Among the most overt negative changes experienced by man and experimental animals under conditions of weightlessness are the loss of skeletal mass and attendant hypercalciuria. These clearly result from some disruption in the balance between bone formation and bone resorption (i.e., remodelling), but precisely what this disruption is and how it might occur have not been established. Recently, a body of information has accrued indicating that the osteoblast plays an important role in bone resorption as well as bone formation and may hold the cellular "key" to understanding the response of the skeleton to conditions of weightlessness. In the present study, the clonal osteoblastic cell line, UMR 106-01, as well as sections of bone, have been used to investigate the regulation of several proteins whose expression is central to both bone formation and bone resorption; these are Type I collagen (the major organic constituent of bone), collagenase and collagenase inhibitor. Expression has been monitored at the protein level using a combination of techniques including the radiolabelling of the proteins, gel electrophoresis, ELISA assays and immunohistochemical staining, and subsequently at the level of RNA, principally by Northern blot analysis. This project will shed some light on the comprehensive role of the osteoblast in the remodelling process, and, in so doing, provide some insight into how the process might be disrupted under conditions of zero gravity.

Hormonal Regulation of Collagen Synthesis

This section has been completed with the conclusion that parathyroid hormone (PTH) causes approximately a 40% decrease in collagen synthesis by UMR 106-01 cells. The data are part of a publication which appeared in the February, 1989 issue of *Molecular Endocrinology*.

Regulation of Collagenase Synthesis and Turnover

Turnover of collagenase in the media of UMR cells has been shown to be cell-mediated and rapid, with complete removal of 100 ng/ml enzyme 8 hr after its addition. We have demonstrated that this mechanism is saturable and are attempting to radio-iodinate the enzyme in order to investigate the existence of a cell-surface receptor. It has become very clear that control cells have a greater ability to remove the enzyme from the extracellular environment than PTH-treated cells, suggesting the down-regulation of the putative receptor by PTH.

UMR 106-01 Collagenase cDNA Clone and Analysis of Collagenase mRNA

Northern blot analysis was conducted on mRNA from UMR 106-01 cells cultured with PTH for various times. Using oligonucleotide probes, we determined that the mRNA of the rat collagenase gene in UMR cells is approximately 2.9 kilobases in size and maximal expression occurs about 4 hr after treatment of the cells with PTH. Poly(A^+)RNA from UMR cells treated with PTH for 4 hr was used to prepare cDNA libraries in the *Eco* R1 site of Lambda ZAP. Screening of this library with oligonucleotide probes resulted in one 2.5 kbp partial rat collagenase clone. Most of the coding sequence is contained in a 1 kbp subclone (UMRco130) which was used to generate probes for Northern blot analysis and will be used to rescreen the same library to obtain the remaining

5' portion of the rat collagenase gene. Examination of 0, 4 and 24 hr PTH-treated UMR RNA showed approximately a hundred-fold induction of collagenase mRNA 4 hr after PTH treatment. The size of the message was identical to that observed using the oligonucleotide probes. The results indicate the existence of an extremely interesting system to analyze PTH action on the osteoblast. The availability of the partial clone will mean we can undertake studies to dissect out this mechanism and also use the probe to pinpoint the cells in bone which are transcribing the collagenase message. This will be extended to an examination of the abundance of collagenase under various conditions, such as in zero gravity.

Culture of Cells in Roller Bottles versus Stationary Flasks

We have been able to conclude that UMR cells change their phenotype depending on their environment. In roller bottle culture, the cells synthesize greater amounts of collagen and collagenase inhibitor and suppress the secretion of collagenase compared with the amounts observed in stationary culture. This would suggest that either gravitational or shear forces can affect the behavior of osteoblasts.

Immunohistochemical Detection of Collagenase in Sections of Bone

Frozen sections near the sagittal suture (an area of high turnover) of 1, 7, 14, and 21 day old rat calvariae were stained for collagenase by immunohistochemistry. The amount of collagenase increases postnatally and is greatest around 14 days (a time when brain growth is maximal in the rat and, hence, cranial remodelling is at its highest). Collagenase was associated with matrix and restricted to an alkaline-phosphatase staining endocranial zone. We conclude that the enzyme is secreted by a select population of osteoblasts. Preliminary data using adult rats indicates the presence of collagenase in the same restricted endocranial matrix-associated region.

Accomplishments

(1) Demonstrated that the neutral metalloprotease, collagenase, is not only secreted by osteoblastic cells but is also removed from the media by a saturable cell-mediated event.

(2) ***Isolated and sequenced a cDNA clone for rat collagenase from an osteoblastic cDNA library.***

(3) ***Demonstrated that PTH induces collagenase mRNA 100-fold after 4 hr of treatment, thus establishing a highly sensitive system to examine PTH action.***

(4) Obtained data that roller bottle culture causes osteoblastic cells to produce greater amounts of proteins associated with bone formation and lesser amounts of those associated with the process of bone breakdown.

(5) ***Observed collagenase staining in rat calvariae, with the greatest amounts seen at the time when greatest remodelling of the cranium occurs.***

Significance of the Accomplishments

Finding #1: Treatment with PTH appears to change the phenotype of the osteoblast from a matrix-synthesizing cell to one actively involved in the resorption process. Collagen synthesis declines while production of enzymes associated with matrix removal increases. This situation may resemble that seen in situations of weightlessness where bone formation is perturbed. Nevertheless, the cell appears to exert tight control over the amount of

collagenase in the extracellular medium by rapidly re-extracting it *via* a putative cell-surface receptor.

Finding #2: The availability of a probe for rat bone collagenase gives us the ability to embark on a range of experiments as well as to obtain the complete amino-acid sequence for the protein. We are preparing, now, to undertake experiments using this probe to pin-point the cells in bone synthesizing RNA for this protein. We will also use the cDNA to assess the effects of weightlessness on transcript abundance in bones from animals subjected to zero gravity.

Finding #3: One hundred-fold induction of collagenase mRNA by PTH is suggestive of an effect of the hormone at the gene. This system will be very useful to dissect the signal transduction pathways involved in PTH action on the osteoblast.

Finding #4: The effect of roller bottle culture compared with stationary flasks on osteoblastic cells indicates their responsiveness to motion and/or gravity and that forces such as these are necessary to maintain the formative phenotype.

Finding #5: Suggests that the osteoblast produces collagenase under conditions of rapid remodelling. This will also form the basis of studies to be performed on tissues from rats flown on the COSMOS biosatellite.

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GRAVITATIONAL EFFECTS ON EARLY DEVELOPMENT IN AMPHIBIAN EMBRYOS

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Description of Research

There are two major research goals being studied in this research project. The first involves the effects of an altered gravitational environment on the early patterning events in amphibian embryos. The second goal is a study of the amphibian embryo's ability to monitor for and correct developmental abnormalities caused by environmental stresses such as unusual gravitational forces. Amphibian embryos are being used in these studies because of the ease of monitoring for developmental defects during the course of early development. The general results and molecular probes developed from these studies should be applicable to mammalian development where observational analyses are much more difficult during the developmental periods of interest.

Studies on early patterning events, the first major goal, is further divided into two parts. One part examines how Earth's gravity is normally used to orient cytoplasmic and cortical rearrangements during development. The effects of these rearrangements during normal development in Earth's gravity is being examined using probes which are specific for several molecular events occurring during the patterning of the neural system. The neural axis is one of the earlier manifestations of the general axis and contains very precise spatial patterns which can be assayed at many levels of complexity. The corollary to the question of the effects of cortical and cytoplasmic positions during normal development is how abnormal cytoplasmic rearrangements, due to various situations of altered gravitational environments, affect the development of the neural pattern. This question is also being examined using molecular probes to follow the course of neural development in embryos subjected to varying centrifugal forces during early development.

The second portion of the study on early patterning events examines the mechanisms which drive the patterning process and how gravity might affect the spatial locations of tissues whose sequence of signaling events ultimately dictate the neural pattern. Several authors have already demonstrated that a nonaliened orientation of amphibian embryos, relative to Earth's gravity, can override the embryos' normal mechanisms, cued by the sperm entry point, to position the neural axis. Presumably, this occurs by cytoplasmic rearrangements so that the blastopore lip, the first and dorsal-most portion of the gastrulating mesoderm, is now established in a gravity-driven position. This laboratory has initiated a series of studies to determine: (1) what are the mechanisms for patterning the neural system and, (2) what tissues are involved and how do they affect this process? These are important questions because if one wants to understand how gravitational stresses affect the final morphology of neural development, then it is necessary to know something about the sequence of events which leads to the observed morphology. Therefore, we want to know the tissues and mechanisms that are normally responsible for establishment of neural pattern so that when the neural pattern is altered by gravitational stresses, we will know what mechanisms, tissues, or cellular events gravity might have affected to produce the altered pattern. Our approach is to use tissue specific molecular probes to study which tissues are responsible for inducing and establishing the neural pattern and how they do it. The initial set of studies on this problem has been completed and published (Savage and Phillips, 1989).

The second major direction of this project follows an observation we made concerning the amphibian embryo's ability to regulate or correct for neural pattern deficiencies caused by gravitational stresses. We were able to observe this phenomena only because we have developed the appropriate molecular probes which allow us to evaluate how the early events in neural pattern formation are proceeding. Early embryos were centrifuged to perturb normal cytoplasmic/cortex relationships relative to their anterior-posterior axis. We find that, within limits, the early embryo can correct for these abnormal situations and produce a morphologically normal embryo. Preliminary experiments suggest that presumptive neural ectoderm cells underwent some form of corrective change during mid-cleavage which effectively "rescued" them back to the correct developmental pathway. We are interested in knowing how far the embryo can be perturbed or stressed, using altered gravitational environments as the force for stress, and still allow for "normal" development through the process of regulation. We are also studying the mechanisms whereby embryos monitor for normal development and how they might correct the emerging patterns and how much abnormality they can correct for.

Accomplishments

(1) We have used a monoclonal antibody as a spatial marker for neural development. We have found that ***cells destined to become neural are biased in this direction much earlier in their development than previously believed.*** This initial bias is very likely to be the result of cytoplasmic mixing, which is subject to gravitational influences, occurring shortly after fertilization.

(2) We have found that ***a small group of cells, which eventually form the initiation site for gastrulation movements, have the ability to pattern subsequent neural development.*** The spatial position of this same group of cells is subject to gravitational perturbations between fertilization and first cleavage.

(3) Using centrifugation to simulate gravity-driven cytoplasmic rearrangements, we have demonstrated that ***amphibian embryos can regulate for certain amounts of gravitationally induced stress and produce normal embryos.***

Significance of the Accomplishments

These studies will become important issues in the raising and care of animals in an extraterrestrial environment, as well as defining potential problems for human reproduction outside of the influence of Earth's environment. We should gain an understanding of what specific kinds of problems embryos will face in terms of development. We will have a better idea how much environmental stress early embryos can tolerate and we can also begin to select animals with specific characteristics for regulation to tolerate and correct for such stresses. All of these studies use molecular biology techniques to determine how embryos might use alternative pathways to achieve normal morphology in an abnormal or perturbed environment.

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CELL KINETIC AND HISTOMORPHOMETRIC ANALYSIS OF MICRO-GRAVITATIONAL OSTEOPENIA

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Description of Research

Differentiation of cells supporting bone formation is inhibited by decreased skeletal loading and microgravity. The long-range goals and objectives for this research are: (1) to define the cellular mechanism of osteoblast (bone-forming cell) production and (2) to determine how this process is suppressed in microgravity. DNA labeling (^{3}H-Thymidine uptake), mitotic activity, and nuclear size are used as indices of the proliferation and differentiation aspects of osteoblast production.

There are three kinetically and/or morphometrically distinguishable cell types in the osteoblast histogenesis sequence: (1) self perpetuating, less differentiated precursor cells (**A** type), (2) committed osteoprogenitor cells (**A'** type), and (3) preosteoblasts (**C+D** cells). The osteoblast (**Ob**) histogenesis sequence is **A** $\rightarrow$ **A'** $\rightarrow$ **C** $\rightarrow$ **D** $\rightarrow$ **Ob**. Increase in nuclear volume (**A'** $\rightarrow$ **C**) is a morphological manifestation of change in genomic expression (differentiation). This method is applicable to all skeletal sites tested, i.e., periodontal ligament (PDL), tibial metaphysis, mandibular condyle, and mandibular periosteum.

Research conducted in the past year focused on further definition of osteoblast production in rat molar PDL. The following principal questions were addressed: (1) Effect of 3 days of simulated weightlessness (SW) on circadian rhythm of DNA synthesis among osteogenic cells? (2) Influence of 12.5 days of spaceflight on cell kinetics of osteogenic cells? (3) Role of prostaglandins synthesis in mechanical induction of preosteoblast formation (**A'** $\rightarrow$ **C**)?

Accomplishments

Flight Data - Cosmos 1887

(1) Compared to synchronous control animals, spaceflight followed by ~55 hr recovery period resulted in a 40% decrease in the **A+A'** cell population ($p \leq 0.05$) and a 42% increase in the **C+D** cells ($p \leq 0.05$).

(2) The total cell density of PDL fibroblast-like cells within 25 μm of the bone surface was increased by 39% in the flight groups ($p \leq 0.05$).

(3) ***Recovery of preosteoblast formation occurred despite a highly significant degree of physiological stress*** (as measured by changes in adrenal weight).

Simulated Weightlessness (SW) - Unloaded Rat Model

(4) Unloading resulted in a 35% decrease in preosteoblast (**C+D** cell) formation in the PDL of animals sampled in the evening (9 p.m.).

(5) The ***fractional distribution of osteoblast precursor cells and the number of cells undergoing DNA synthesis in the (PDL) of SW animals***

exhibited a circadian rhythm pattern that was 12 hr out of phase with the pair-fed control group.

(6) There was a significant correlation (r = 0.80) between the fractional distribution of cell types and the number of cells undergoing DNA synthesis (S-phase cells).

Indomethacin and Osteoblast Histogenesis

(7) The indomethacin treated group of rats had greater than a two-fold increase in the numbers of **A+A'** cells and a 53% increase in the **B** cell population when compared with a control, orthodontically stressed group. However, both the **C** and **D** cell populations were reduced with respect to the control values by 23% and 43%, respectively.

(8) Indomethacin treatment also resulted in approximately a 55% decrease in the fraction of PDL cells labeled with ^{3}H-thymidine.

Significance of the Accomplishments

Finding #1: This burst of preosteoblast formation indicates a strong postflight recovery of osteoblast histogenesis in the periodontal ligament.

Finding #2: These data indicate a movement of osteogenic cells toward the bone surface which is also consistent with a strong recovery of osteogenic potential.

Finding #3: The recovery of osteoblast histogenesis occurred despite chronic stress (Figure 1a). This is a particularly significant result because bone formation is usually suppressed by corticosteroids. ***Postflight recovery of osteogenic potential is a powerful physiological phenomenon.***

Finding #4: Confirmation of a decrease in preosteoblast numbers at 9 p.m. but not at 9 a.m. indicates that the p.m. period is the most reliable sampling time for demonstrating the transient suppression of osteogenesis at 72 hr after initiation of SW.

Finding #5: These data indicate that the transient suppression of preosteoblast formation within 72 hr after initiation of SW is associated with disturbance of circadian rhythm of osteogenic cells.

Finding #6: Despite the size of the precursor cell (**A+A'**) and preosteoblast (**C+D**) compartments, the number of proliferating cells remained relatively constant. This indicates a ***relatively constant control of cell turnover under resting conditions*** which is independent of the total number of a particular cell type.

Finding #7: These data indicate that the ***major rate-limiting step in preosteoblast formation is probably mediated by prostaglandins and the block is occurring after induction of the A' → C shift*** (Figure 1b).

Finding #8: Suppressed progression of osteoblast precursor cells to osteoblasts supports the conclusion that ***mechanical induction of preosteoblast formation (A' → C) is prostaglandin mediated.***

OSTEOBLAST HISTOGENESIS PATHWAY

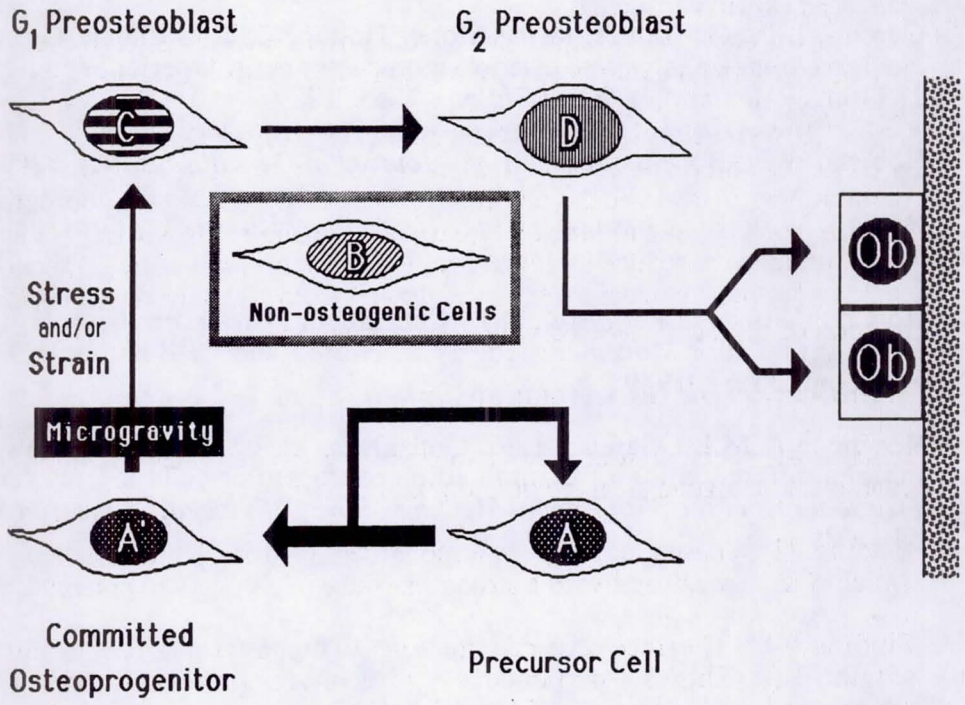

Figure 1. Schematic diagrams of the osteoblast (Ob) histogenesis pathway, demonstrating the sequence of progressively more differentiated osteogenic cells (A, A', C, D). B cells are present but do not particpate in osteoblast production. Figure 1a. Schematic drawing of the microgravitational block of osteoblast histogenesis.

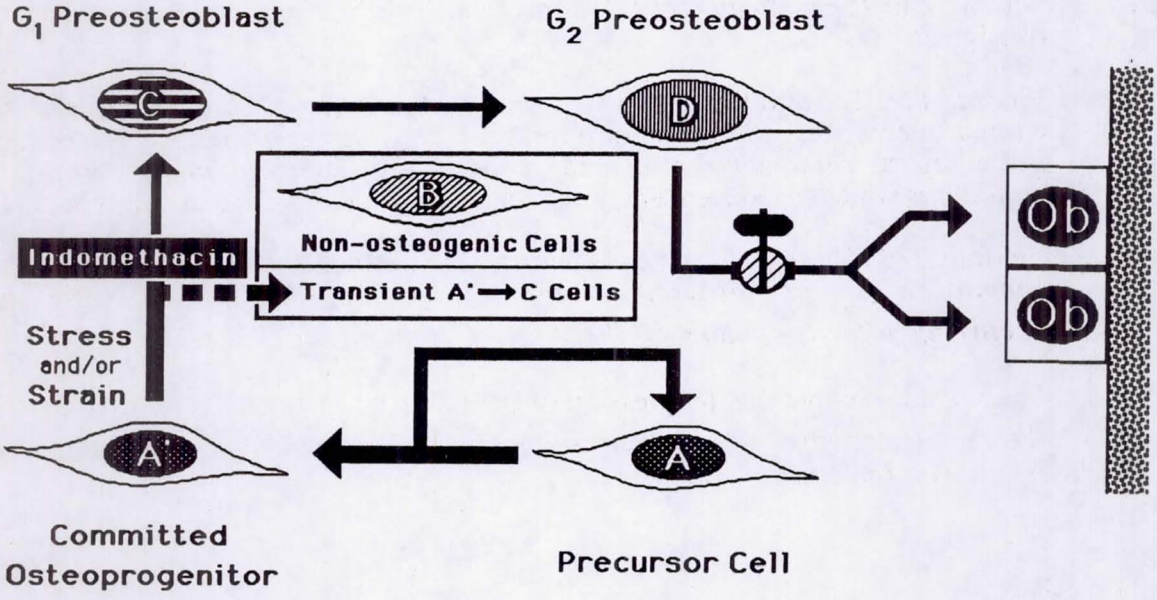

Figure 1b. Schematic drawing of the indomethacin block of osteoblast histogenesis

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STRUCTURE AND FUNCTION OF MAMMALIAN GRAVITY RECEPTORS

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Description of Research

The long-range objective of this research is to understand vestibular and other neural adaptations to microgravity. To achieve this objective our immediate goal is to produce 3-dimensional (3-D) reconstructions of macular receptive fields and small parts of the neural network for interpretive purposes.

Reconstructions begin with serial sections studied and photographed in a transmission electron microscope. For the present research, fifth sections of a 570 section series cut through the utricular macula of a Sprague-Dawley rat were used. Neural elements were traced from montages made from the electron micrographs. The tracings, made on acetate sheets, were digitized into a PC, and the data were transferred to an IRIS workstation for 3-D reconstruction as shaded solids. Some of the software for shaded solid reconstruction was available on the IRIS, and the rest of the software, including that necessary for animation and recording on film, was developed in this laboratory.

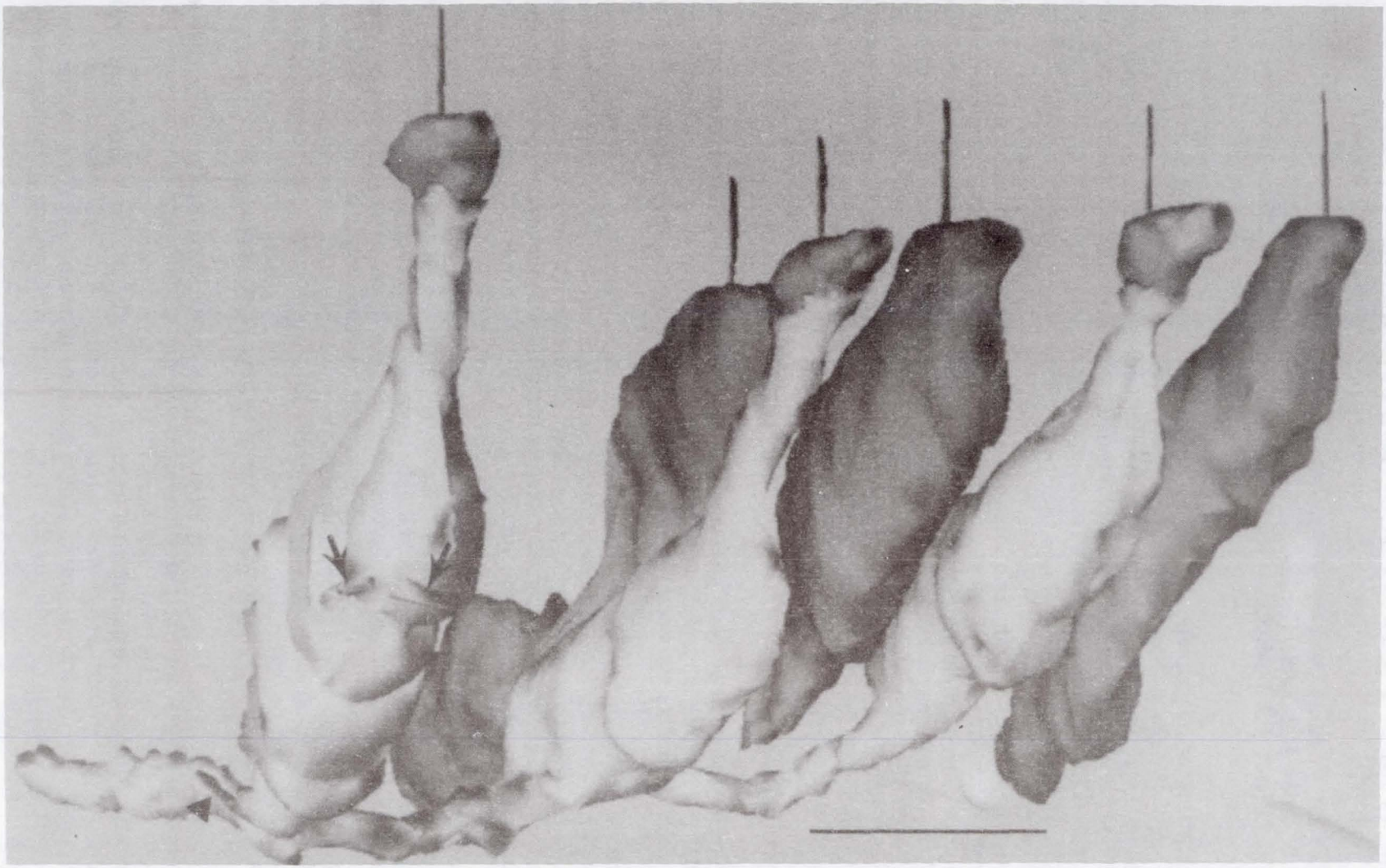
Accomplishments

During the past year we have generated 18 three-dimensional (3-D) reconstructions of macular receptive fields and three portions of the neural network. The connectivities of more than 200 cells have been mapped. The reconstructions show that ***both type I and type II hair cells comprise receptive fields,*** and that ***type II hair cells distribute their output to terminals of up to 4 nerves. No two fields are identical.*** Nevertheless, ***three basic configurations of fields have been identified.*** When only a vestibular nerve fiber terminates in a single calyx, the receptive field is rounded at the macular surface. Nerves that bifurcate and have two terminal calyces tend to be oblong, and nerves with three branches and three terminal calyces are elongated.

There are three kinds of nerve patterns as well. In our series, 82% of the nerves have long unmyelinated preterminal segments (U-type). U-type nerves usually have three branches, three terminal calyces, and highly elongated fields (Figure 1). Another 12% of the nerves have short unmyelinated preterminals (M/U). These nerves may end in a single calyx but, more typically, they bifurcate into short branches and have oblong fields. The remaining 6% of the nerves are myelinated to the calyx (M-type), have single calyces and rounded fields. In all but extremely rare examples (two in our extensive maps), ***type II hair cells synapse with only one of the two or three calyces of a given nerve*** even though they synapse with one or more calyces of other nerves.

A further finding is that ***U-type nerves have many collaterals.*** While ***some collaterals spring from the parent nerves and are usually efferent*** in type (they have vesiculated terminals), ***most collaterals arise from calyces*** (Figure 1). ***Calyceal collaterals*** terminate on type II hair cells and ***are either afferent, efferent, or of mixed properties morphologically.*** The ***collaterals add to the complexity of the neural network.*** Thus, portions of the network consisting of only U-type nerves are more complex appearing than those which contain all three kinds of nerve patterns.

STRUCTURE OF U-TYPE NERVES



Black and white rendering of a reconstruction of a U-type nerve (thin object at lower left) with three branches and three calyces (all shown in white). The heads of the type I hair cells (shaded in gray) project from the tops of the calyces. Type II hair cells are the shaded objects lying adjacent to the calyces. The thread-like extensions from the heads of the hair cells are the kinocilia. The arrowhead at lower left indicates an efferent-type collateral emerging from the parent nerve, and the two arrows indicate collaterals originating from the calyx at the left. (Efferent terminals are not illustrated in this figure). Bar (lower right) =10 μm.

The existence of efferent-type nerve and calyceal collaterals means that there is an extensive, intrinsic system of efferents to modify information processing as it is taking place.

Type II hair cells have more synapses with nerve terminals than do type I cells, which synapse only with calyces. ***The number of synapses varies from cell to cell and cannot be predicted*** on any grounds yet known.

Significance of the Accomplishments

The results indicate that maculas are morphologically organized for weighted, parallel distributed processing of information, and that the processing is subject to intrinsic modulation by calyceal and nerve collaterals. Spatiotemporal features of information processing are important to neural responses and to neural coding. Each calyx appears to be a processing unit for the territory of its receptive field, and adjacent fields overlap. The fact that no two receptive fields are identical strongly suggests that wiring is not under strict genetic control, but that an element of randomness is introduced developmentally. This randomness, together with built-in redundancy, likely contributes to the robustness of the sensory endorgans and endows them with adaptability to new linear acceleratory environments such as those encountered in space, on the moon, or on other planets (such as Mars).

Publications

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EFFECTS OF WEIGHTLESSNESS ON *AURELIA* EPHYRAE DIFFERENTIATION AND STATOLITH SYNTHESIS

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Description of Research

The long-range goals of this research are (1) to discover the role(s) of gravity on the behavior and development of *Aurelia* ephyrae (tiny jellyfish) and on their graviceptor structures (rhopalia) and (2) to discover the effects of microgravity on ephyrae and graviceptor development after short-term (8-day space shuttle flight) and long-term (space station and biosatellite) exposure to a microgravity environment.

Specific objectives are: (1) to determine whether the microgravity of space will modify the development of ephyrae from polyps; the development of the graviceptors of ephyrae; the mineralization and/or demineralization of statoliths of rhopalia; or the swimming/pulsing behavior of ephyrae; (2) to discover, by comparing the features listed above in ephyrae that develop in space with those of ephyrae that develop on Earth, the roles that gravity plays in the development of ephyrae, their graviceptors, and their behavior on Earth; (3) to develop a method for maintaining rhopalia in nutrient media for 1-3 months in order to study development of more mature rhopalia (than those found in ephyrae) on Earth and in space; and (4) to compare behavior of ephyrae with and without rhopalia.

Accomplishments

(1) Effect of graviceptor excision on *Aurelia* ephyrae swimming/pulsing behavior at 1 g and during parabolic flight (in collaboration with Dr. R. Phillips and A. Kostas, Colorado State University): The swimming/pulsing behavior of ten *Aurelia* ephyrae was videotaped before and after the rhopalia (graviceptors) were removed. (Ephyrae with rhopalia removed by this method were examined with the SEM to verify absence of rhopalia.) Two controls were videotaped before and after small cuts were made in their stomach area. The rhopalia-free ephyrae and controls were videotaped immediately after cutting and approximately 4, 24, and 60 hr later. Derhopaliaized (DR) ephyrae did not regain their ability to swim, although they were able to pulse occasionally. After 96 hr, the ephyrae were flown on a NASA parabolic flight which flew 40 parabolas. The DR ephyrae did not respond to g force changes to near 0 g or even to hyper-g (ca 1.8 g). Controls, however, responded to g changes in various ways. Some of them swam in loops upon changes in g force (usually at the onset of near zero g) before becoming immobilized; others became immobilized immediately. Most controls resumed pulsing after a brief time and pulsed more rapidly in hyper g.

(2) Culture of ephyrae and growth of their rhopalia in a liquid nutrient medium: Three experiments were performed in which ephyrae were cultured in several dilutions of a sea water-based liquid nutrient medium. The growth and maturation of their rhopalia into structures resembling medusa rhopalia was achieved. This method, for the first time, allows medusa rhopalia to be grown in aseptic media in small, clean, tissue culture bottles. This method provides better synchronized rhopalia growth than can be achieved by growing medusae in large tanks with living food (brine shrimp). Nutrient medium-grown ephyrae developed large rhopalia (Figure 1) with a second ocellus within ten days and showed early touch-plate formation within five weeks. Most dramatic, however, was the

formation of large numbers of statoliths which are much larger than statoliths of ephyrae that are not grown on nutrient medium (Figure 2).

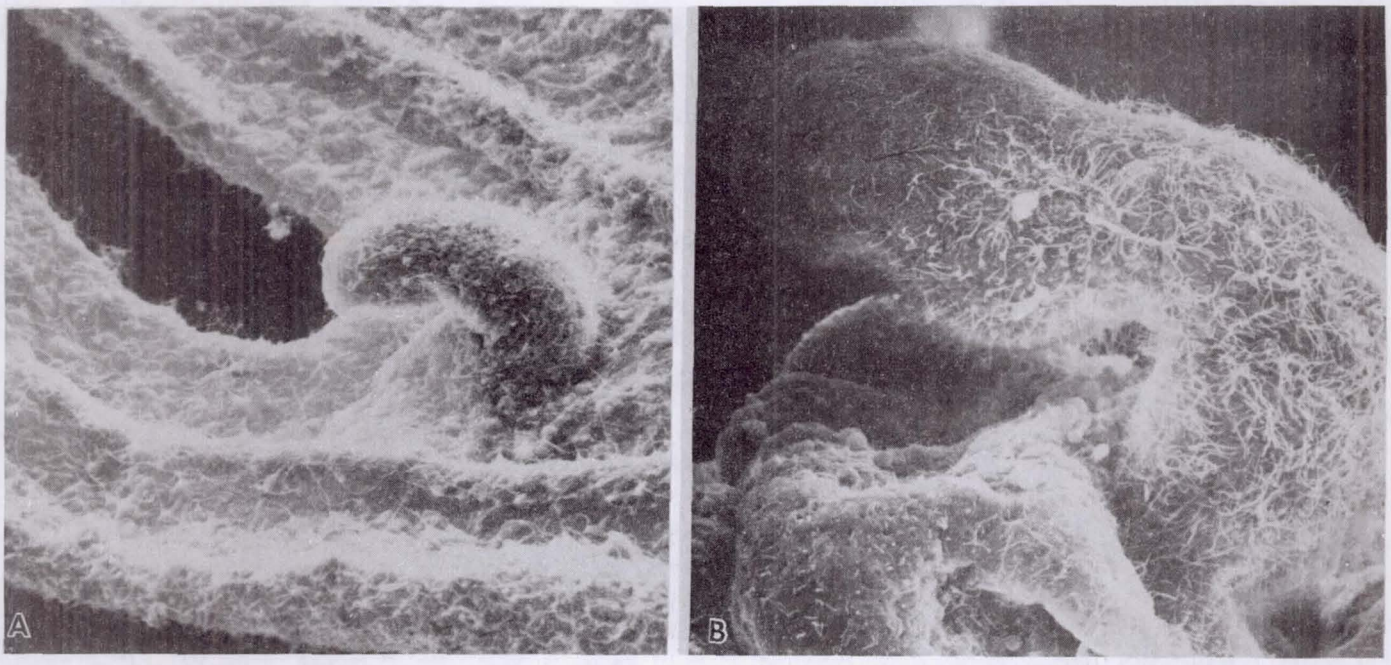

Figure 1a. Rhopalium of *Aurelia* ephyra. 1b. Rhopalium of ephyra grown in nutrient medium for five weeks.

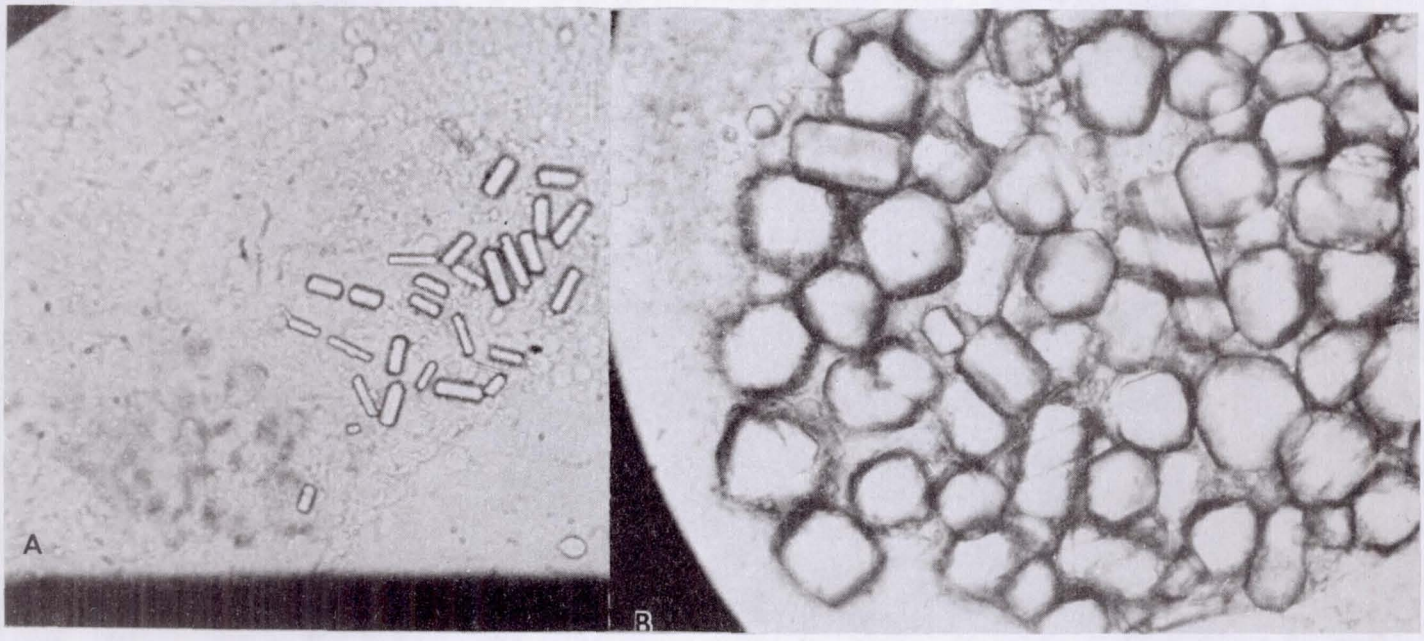

Figure 2a. Statoliths of ephyra. 2b. Statoliths of ephyra grown in nutrient medium for seven weeks.

Significance of the Accomplishments

Our research is directed toward learning as much about *Aurelia* ephyrae behavior and development as possible in the time interval before the Jellyfish Experiment is flown on the SLS-1 shuttle flight (currently scheduled for August 1990). The emphasis this year was placed on experiments which will contribute to a better understanding of the role of the graviceptors in ephyra swimming/pulsing behavior and to learn, through comparison with medusa graviceptors, those features which are essential for graviceptor function.

Finding #1: Previous information concerning the function of graviceptors in *Aurelia* was derived from studies of medusae. Ephyrae, however, lack a bell and therefore move and orient differently than medusae. The importance of the graviceptor structures for ephyra swimming and orienting was not known although graviceptors were known to be involved in pulsing regulation. Our ephyra study was the first of its kind in which the graviceptors were removed from the tiny animals and their behavior recorded in the same organism before and after excision. Ephyrae without graviceptors could not swim and pulsed only rarely as they sank to the substratum. ***The results clearly demonstrate the importance of the graviceptors of ephyrae for swimming activity as well as for rhythmic pulsing. The hyper-g challenge to the DR ephyrae during parabolic flight demonstrated that, without graviceptors, the organism could not be stimulated to pulsing/swimming activity.*** Clearly, the organisms do not have compensatory structures apart from the graviceptors which respond to changes in g force.

Finding #2: ***For the first time, the growth and maturation of ephyra graviceptors into medusa-type graviceptors was achieved in a simple liquid nutrient medium. The growth of the graviceptors was more rapid and better synchronized than can be achieved by the culture of the organisms in a large, aerated tank with living food.*** This simple growth method will be used to obtain mature rhopalia which can be compared with ephyra rhopalia to better understand those features which are important for gravity perception. Further, the method can be applied to jellyfish microgravity experiments in long-term unmanned flights, in which the development of the touch-plate and large statoliths of the graviceptors can be compared on Earth and in space.

Publications

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SKELETAL MUSCLE METABOLISM IN HYPOKINETIC RATS

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Description of Research

This research concerns the mechanisms of atrophy and metabolic alterations associated with lack of load-bearing (unloading), which may occur with prolonged bedrest or weightlessness. To further understand this muscle wasting, comparisons are made with measurements in muscles whose nerve supply is interrupted. Our work has dealt primarily with carbohydrate and protein metabolism in muscle.

For carbohydrate metabolism, we finalized measurements of the physiological activity (i.e., activity at the physiological concentration of the effector) of glycogen synthase and phosphorylase, and their relationship to changes in glycogen concentration following reloading. The influence of isoproterenol (beta-adrenergic agonist) on glycogen metabolism, glucose uptake and glucose-6-phosphate production, as well as beta-adrenergic binding capacity, were compared in unloaded and control muscle. Studies of protein metabolism included testing of an oral glucocorticoid antagonist for effects on *in vivo* protein metabolism and whether abolishing circulating insulin can prevent the decline in proteolysis after 3 days of unloading.

Accomplishments

(1) **Carbohydrate Metabolism**

(a) ***With reloading, changes in the physiological activities of glycogen synthase and phosphorylase can generally account for alterations in glycogen content.*** Glycogenolysis during 15 min to 2 hr of reloading is associated with increases in glucose-6-phosphate and glycogen phosphorylase activity measured at fresh muscle AMP concentrations. While glycogenesis from 2 hr to 4 hr correlates well with enzyme activities, between 4 hr and 24 hr increases in glycogen concentration are uncoupled from synthase activity. Between 24 hr and 72 hr, glycogen decreases to control values, possibly initiated by increased phosphorylase activity at 24 hr.

(b) ***Unloading results in an increased isoproterenol (beta-adrenergic agonist) responsiveness for glycogenolysis;*** however, isoproterenol's influence on the rates of glycogenesis, glucose uptake, and glucose-6-phosphate production does not differ between unloaded and control soleii.

(c) ***The beta-adrenergic binding capacity of the soleus increases markedly following three days of unloading.*** The affinity of the beta-receptor for dihydroalprenolol (beta-adrenergic antagonist) is not influenced by unloading.

(2) **Protein Metabolism *In Vivo***

(a) ***Blocking the glucocorticoid receptor in unloaded animals does not change the effect of unloading on muscle protein content or the response of protein synthesis relative to controls.***

(b) ***Blocking the glucocorticoid receptor leads to less atrophy in unloaded soleus but greater growth in control muscle due to slower protein degradation.***

(c) ***The decline in protein degradation noted after day 3 of unloading is abolished in diabetic animals.***

(d) ***Other membrane proteins (e.g., nucleotidase) besides the insulin receptor may be spared during unloading-induced atrophy.***

Significance of the Accomplishments

Finding #1: Finding 1a suggests that *in vivo* activities of glycogen synthase and phosphorylase are best measured at physiological effector levels. However, not all changes in effector levels or glycogen are directly associated with altered enzyme activity. Findings 1b and 1c indicate that while the enhanced isoproterenol responsiveness for glycogenolysis may be accounted for by increased beta-adrenergic sensitivity, other aspects of carbohydrate metabolism are not similarly affected by the augmented beta-adrenergic binding capacity of unloaded muscle.

Finding #2: (a,b) Glucocorticoids clearly are not the cause of atrophy in unloaded soleus muscle though this hormone may exacerbate the atrophy. (c) Increased sensitivity to insulin which occurs on day 3 in the unloaded soleus may diminish muscle atrophy at this time through a slowing of proteolysis.

Publications

Henriksen, E.J., Satarug, S., Tischler, M.E., and Fürst, P. Responses of lysosomal and non-lysosomal proteases to unloading of the soleus. In: *Proteases II. Potential Role in Health and Disease* (ed. by W.H. Hörl and A. Heidland). New York: Plenum Press, pp. 235-242, 1989.

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EFFECTS OF MICROGRAVITY ON THE STATOCYST OF *APLYSIA CALIFORNICA*

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Description of Research

The *Aplysia* statocysts are spherical gravity receptors, their wall being comprised of 13 large receptor cells, with cilia projecting into the cyst lumen, and numerous small supporting cells. The test mass which senses gravitational forces is a collection of separate stones, or statoconia, within the statocyst lumen. The *Aplysia* statocyst is a favorable model system in which to quantitatively study the physiology, anatomy, and development of gravity receptors. Since there are relatively few stones (compared to mammalian species) and they are within soft tissue, rather than the hardest bone in the body (as is the case in mammals), it is relatively easy to quantify the number of stones at different developmental stages. This will be valuable in quantifying the effects of microgravity on the development of this gravity-sensing organ. There is concern that during deep-space missions, in which animals or humans would be conceived and born in microgravity, the gravity receptors may not develop normally. Such organisms could have serious problems in later adapting to a 1 g environment.

In the past year we have investigated the time course and site of production of statoconia in embryonic, newly-hatched, early post-metamorphic, juvenile, and adult *Aplysia*. We have used light and electron microscopy to quantify the number of statoconia in specimens of different age and weight. The statoconia have a complex structure, consisting of alternating layers of membrane and calcification (aragonite). Image-analysis techniques have been used to quantify the distribution of statoconia diameters in animals of different size, in an effort to determine whether stones increase in size after they are formed. Exposure to microgravity could affect both the initiation of statoconia production as well as their growth.

Accomplishments

(1) ***The time course of production of statoconia has been determined to proceed as follows:***

(a) ***A single statolith is present from later embryonic stages, through the larval stage and early after metamorphosis*** (Figure 1).

(b) ***Multiple statoconia production begins when the juvenile animal reaches approximately 1.2 mm in length.***

(c) ***Once multiple stone production is initiated, at approximately 60 days of age, additional stones are added throughout life.***

(2) The size distribution of statoconia can be described as follows: (a) The single statolith ranges from 6 μm in diameter in the embryo to 12 μm in early post-metamorphic animals. (b) Multiple statoconia in early post-metamorphic animals of 1-2 mg weight range from 2 to 8 μm in diameter. (c) Multiple statoconia in juvenile animals between 0.1 and 0.5 g range from 2 to 18 μm in diameter, while those of adults between 170 and 300 g are only slightly larger, ranging from 3 to 20 μm.

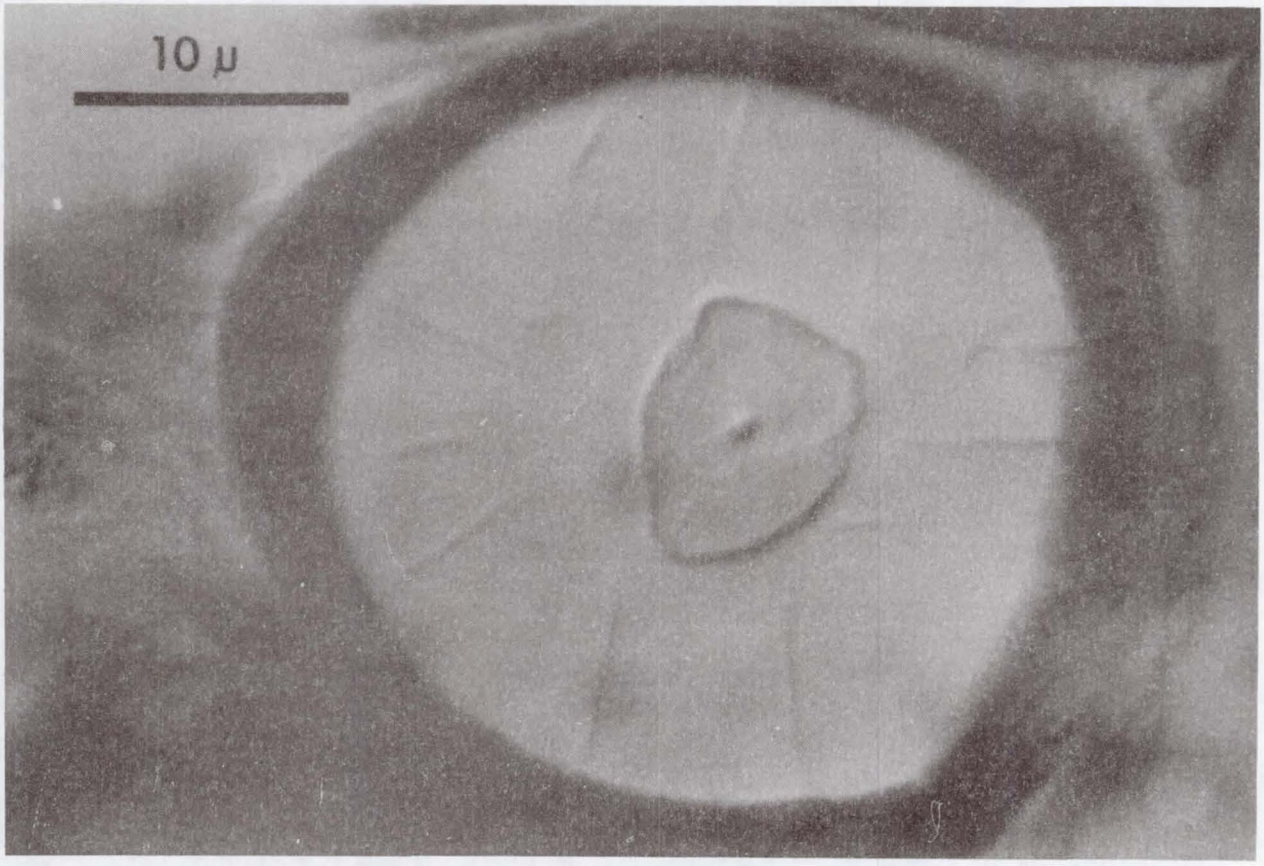

Figure 1. Photomicrograph of a 4 μm thick section through an early post-metamorphic *Aplysia* statocyst. This animal was 60 days post-hatching and weighed less than 1 mg. At this stage the cilia from all 13 receptor cells support the single statolith in the center of the cyst lumen, as opposed to the hundreds of statoconia that fall to the bottom of the lumen in an adult animal (See Wiederhold summary in *1987-88 NASA Space/Gravitational Biology Accomplishments*, pp. 167-169).

(3) ***In all animals with only a single statolith, the receptor-cell cilia project to the statolith*** (Figure 1). ***Multiple statoconia production begins when the statocyst grows to the size at which the cilia can no longer support the statolith. Thus, all statocysts of 44 μm or greater diameter have multiple statoconia and all smaller statocysts have just a single statolith.***

(4) Embryonic and early larval statocysts have many fewer receptor-cell cilia than do juveniles and adults. The supporting cells also have a greater area facing the cyst lumen.

(5) ***Transmission electron micrographs of early post-metamorphic statocysts have demonstrated that the multiple statoconia are produced in the supporting cells.***

Significance of the Accomplishments

These results have pinpointed the stage of development at which multiple statoconia production is initiated. This event is triggered when the receptor-cell cilia can no longer support the statolith in the center of the statocyst lumen. Presumably, the statolith then falls to the bottom of the cyst and interacts with only a few receptor cells, rather than with all 13 simultaneously. Either this change in pattern of stimulation, or perhaps direct gravitational action of the statolith on the lumenal surface of the supporting cells, triggers the supporting cells to start producing statoconia. Either of these mechanisms suggests that if the animals were in microgravity when the statocyst reaches 44 μm in diameter, the initiation of multiple stone production would be either prevented or delayed. Since this stage can be easily identified by the length of the post-metamorphic animals, specimens can be selected which should initiate multiple stone formation during a one-week spaceflight mission. These animals would be compared to one group of siblings maintained on a 1 g centrifuge on the spacecraft and another ground-based group. This will tell us whether, in this model system, microgravity affects the development of the gravity-sensing organ.

Publications

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EFFECTS OF SIMULATED MICROGRAVITY AND HYPERGRAVITY ON MAMMALIAN DEVELOPMENT AND DIFFERENTIATION

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Description of Research

The long-range goal of our research has been to assess the effects of altered gravitational environments on mammalian reproduction.

Our research has focused on examining the effects of a simulated microgravity environment on the development and differentiation of mammalian germ cells and early embryos. We previously showed that mouse oocytes rotated on a clinostat exhibited anomalies of the meiotic maturation process. No alterations in the efficiency of fertilizaton were noted. We have extended these studies to include examination of very early embryos. We have now begun to use sensitive molecular markers of normal development to evaluate the effects of our experimental system on early development and on the male reproductive system. This will be useful not only for studying directly the effects of altered gravitational fields, including both microgravity and hypergravity, on mammalian development but also for evaluating biological effects on cells flown in space. In particular, our initial studies have focused on examining the expression of heat shock, or cellular stress genes, of the *HSP 70* and *HSP 90* gene families.

Accomplishments

(1) ***Sub-cellular events in mammalian cells may be affected by simulated microgravity.*** That is, reorientation of mammalian oocytes relative to the gravity vector, through the use of a clinostat, affects meiotic maturation. Oocytes rotated at 100 RPM in the experimental axis revealed an inhibition of achieving metaphase II of meiosis, whereas oocytes rotated in the control orientation did not. This may reflect inhibition of normal chromosome orientation and movement. ***No abnormalities in the appearance of fertilized ova or in the efficiency of fertilization have been observed in ova which were rotated at 100 RPM at the time of fertilization.*** In these experiments, ova which had undergone meiotic maturation *in vivo* were placed with capacitated sperm in the clinostat rotation system. The ova were rotated at 100 RPM (the speed at which meiotic abnormalities had been observed) for 8 hr and were examined for the presence of pronuclei and for any morphological abnormalities.

(2) Although rotated ova appeared normal and fertilization rates were similar between the experimental and control systems, we are interested in evaluating more fully the developmental potential of such rotated ova. Current studies involve transfer of embryos into pseudopregnant mothers in order to assess ***the effect of simulated microgravity on early embryogenesis.*** In these experiments, embryos are embedded on low melting point agarose and immobilized at the center of the axis of rotation. With these conditions and rotation at 100 RPM, ***development up to the blastocyst stages has been achieved.*** Ongoing method control experiments (n=32) involve removing embryos from the culture system when they reach either the two-cell or blastocyst stage and then transferring them into either an oviduct or a uterine horn of a

pseudopregnant female in order to assess implantation and later stages of development. ***A modification of our original embryo transfer technique,*** which entails beveling the transfer micropipet on a microforge, has increased our ability to insert the micropipet into the infundibulum and has raised our successful transfer of two-cell embryos into the oviduct by up to 60% (n=25). In establishing the embryo transfer technique, we have had a 20% success rate (n=15) in obtaining litters from transferred embryos. ***Up to 67% of two-cell embryos (n=15) transferred to an oviduct of a foster female were carried to term.***

(3) An effort has been initiated to ***develop sensitive molecular markers for normal development.*** This will enhance the ability to analyze experiments using the clinostat and most importantly, will greatly enhance the potential analysis of space-flown tissues. As molecular markers of both normal cellular differentiation as well as cellular response to stress, we have chosen to examine the expression of the ***genes for the cellular stress proteins*** (also called heat shock proteins), using ***detection methods at the level of RNA and proteins.*** We have examined the expression of cellular stress genes in germinal cells at the level of protein expression using immunohistochemical techniques. Antibodies to *HSP 70* heat shock proteins obtained from Bill Welsh (University of California, San Francisco) were detected in rotated and control embryos at the blastocyst stage, suggesting constitutive expression of the protein detected by these antisera. More specific antisera, recognizing individual *HSP 70* family members are thus needed for this analysis and are currently being generated. Individual embryos and tissues can be processed and studied at the RNA level by using *in situ* hybridization. Our initial studies have focused on characterizing the expression of the *HSP 70* and *HSP 90* families of cellular stress proteins in reproductive tissues. Our observations have demonstrated that both *HSP 70* and *HSP 90* gene family numbers are under both developmental regulation and stress-induced regulation in the mouse testis and would be useful as sensitive molecular indicators of cellular stress.

Significance of the Accomplishments

Finding #1. The use of embryo transfer will permit us to test the capability of embryos subjected to simulated altered gravity to develop to term and ultimately, to consider implanting early embryos subjected to the microgravity environment of spaceflight upon return to Earth.

Finding #2. Sensitive indicators of normal development and differentiation are needed for assessing the effects of altered gravitational environments on cells. Our studies on the expression of the cellular stress protein genes will be very useful for both ground-based and flight studies. In particular, we will make maximum use of the very precious flight tissue to examine the possible effects of altered gravity or other biological stresses. Moreover, since the effects of gravity can be very subtle at the microscopic level, sensitive indicators of deviation from normal parameters are crucial for utilization of altered gravitational environments as a tool for studying mechanisms of development and differentiation.

Publications

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SPECIAL ACTIVITIES

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SPACE BIOLOGY RESEARCH ASSOCIATES PROGRAM

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The Space Biology Research Associates Program provides a unique opportunity to train individuals to conduct biological research in areas relevant to NASA's interest. To maximize the potential for Space Biology as an emerging discipline, there is a need to develop a cadre of scientists interested in working in this area. This grant was developed to train biologists by offering Research Associate Awards to young scientists. These awards provide opportunities for individuals to work on projects directly related to Space Biology and in laboratories that provide the necessary facilities and a relevant research environment. It is anticipated that these scientists will develop research careers in the evolving discipline of gravitational biology, a focused area of Space Biology. The field of gravitational biology is rapidly growing and its future will reflect the quality and training of its scientific personnel.

The program began on June 1, 1980 with funding to support several Research Associates each year. As of May 13, 1989, 69 annual awards had been made to 43 awardees, of whom 26 have received a second year of funding. On May 13, 1989 the Review Panel recommended six more awards. Table I illustrates the variety of projects, laboratories and institutions to which the 43 Research Associates have been assigned. These scientists represent different disciplines including: zoology, developmental biology, botany, and physiology (animal and plant). In June 1980 there were 19 laboratories participating. Presently there are 59 laboratories in the program.

Many of the Research Associates have been asked to participate in NASA panels, national workshops and national meetings. There have been 111 publications in refereed journals and as many abstracts of papers presented at national and international meetings. Each year in the fall, Research Associates attend the annual meeting of the American Society for Gravitational and Space Biology (ASGSB). The Research Associates are active participants in those meetings, presenting papers and posters along with their senior colleagues. All of the current Research Associates and many of the former Research Associates are members of ASGSB. Research Associates are encouraged to participate in other national meetings in their own disciplines.

The scientists who have completed this program have accepted positions in colleges and universities, with research laboratories and with NASA. An individual listing of the 43 Research Associates is provided in Table I. A more detailed description of the awardees follows Table I.

Table I. SPACE BIOLOGY/RESEARCH ASSOCIATES

ANIMAL PROJECTS

S. BAIN	SUNY, Stony Brook	SKELETAL BONE REMODELING
W. BERRY	U. Louisville	ROLE OF VITAMIN D/IMMUNE FUNCTIONS
M. BINDER	Dartmouth Col.	CARDIAC PATHOLOGY
S. BLACK	U. Cal., Berkeley	AMPHIBIAN DEVELOPMENTAL ORIENTATION
H. BLAIR	Washington U.	CELLULAR/BONE ATROPHY
J. BUCKEY	U. Texas, Dallas	CARDIOVASCULAR RESPONSES
G. BURROWS	N.I.H.	SYNAPTOGENESIS/NEUROPATHOLOGY
D. CLOHISY	Washington U.	OSTEOCLASTOGENESIS/BONE ATROPHY
M. COOPER	U. Cal., Berkeley	OSTEOPOROSIS/BONE ATROPHY
D. DICKMAN	U. Texas, Galveston	SEMICIRCULAR MUSCLE CANALS
S. GLOTZBACH	Stanford U.	NEUROPHYSIOLOGY/CIRCADIAN RHYTHM
C. GOULD	U. Louisville	IMMUNOLOGY/INTERFERON FUNCTIONS
M. GRAY	Tufts U.	MECHANICAL ENVIRONMENT IN BONE ARCHITECTURE
E. GREENFIELD	Washington U.	OSTEOBLAST ROLE/OSTEOCLAST ACTIVITY
T. JONES	U. Cal., Davis	NEUROPHYSIOLOGY/BRAINSTEM POTENTIALS
T. KERR	Wayne State U.	MAMMALIAN VESTIBULAR SYSTEM
D. KLIGMAN	N.I.M.H.	NEURITE EXTENSION FACTOR RESPONSES
K. MCLEOD	SUNY, Stony Brook	ELECTRICAL FIELDS IN BONE REMODELING
D. MEYERS	U. Pennsylvania	GRAVITY PERCEPTION/MICROCRUSTACEAN (FW)
L. MINOR	U. Chicago	RESPONSES OF SECONDARY VESTIBULAR NEURONS
D. MURAKAMI	U. Cal., Davis	HYPERDYNAMIA/VISUAL SYSTEMS
S. PERKINS	Washington U.	VITAMIN D/OSTEOCLAST DIFFERENTIATION
K. POTE	U. Virginia	OTOCONIA Ca BINDING PROTEIN
G. RADICE	Indiana U.	GRAVITY-SENSORS/AMPHIBIAN EMBRYOLOGY
F. ROBINSON	U. Pittsburgh	SENSORY MOTOR PROPERTIES IN UVULA
J. STEFFEN	U. Louisville	GLUCOCORTICOID RECEPTORS & MUSCLE RESPONSES
J. SZILAGYI	Cleveland Clinic	HYPODYNAMIC RESPONSES/ANIMAL MODEL
Y. TORIGOE	U. Cal., Irvine	NEUROPHYSIOLOGY OF GUT

PLANT PROJECTS

S. BARSEL	Michigan St. U.	PLANT CELL PHYSIOLOGY
T. BJÖRKMAN	U. Washington	ELECTRICAL RESPONSES/GRAVITATIONAL SENSITIVITY
T. BROCK	U. Michigan	AUXIN & PROTEIN SYNTHESIS IN GRAVITROPISM
M. DESROSIERS	Michigan St. U.	ELECTRICAL POTENTIAL IN HORMONE TRANSPORT
J. GARAVELLI	Texas A&M U.	PLANT/ALGAE CELL CHEMISTRY
J. GAYNOR	Yale U.	AMYLOPLAST/GRAVITATIONAL SENSITIVITY
M. HARRISON	Washington U.	ENVIRONMENTAL ETHYLENE/GRAVITROPISM
G. JAHNS	U. Houston	LIGNIN BIOSYNTHESIS IN PLANT DEVELOPMENT
K. KUZMANOFF	Stanford U.	ENZYME REGULATORS IN PLANT CELL WALL
M. MATILSKY	Princeton U.	GRAVITY PERCEPTION/COENOCYTE
M. MUSGRAVE	Duke U.	PLANT RESPIRATORY METABOLISM/SPACEFLIGHT
D. REINECKE	Michigan St. U.	IAA DISTRIBUTION/PLANT GEOSENSING
B. SERLIN	U. Texas, Austin	CELL WALL GROWTH/CORN ROOTS
R. SLOCUM	Yale U.	ROLE OF CALCIUM/GRAVISTIMULATION
J. SLONE	Washington U.	AUXIN TRANSPORT/GRAVITROPISM

RESEARCH ASSOCIATE AWARDEES

The awardees are listed alphabetically, including their award term in parentheses, their host laboratory, research title, and current location.

DR. STEVEN BAIN (6/1/88 - 5/30/89) is working on "The Interaction of Skeletal Remodeling With Systemic Disorders: An Obstacle to Extended Space Flight?" in Dr. Clinton Rubin's laboratory at SUNY, Stony Brook, New York.

DR. SARA-ELLEN BARSEL (6/1/87 - 5/30/88) worked on "Molecular and Genetic Phototropism in *Arabidopsis thaliana*" in Dr. Kenneth Poff's laboratory at Michigan State University, East Lansing, Michigan. She is now working for Chemical Abstracts in Columbus, Ohio.

DR. WALLACE BERRY (7/1/88 - 6/30/89) is working on "Lymphokine Producing Capacity of Antiorthostatically Suspended Rats: Relationship to 1, 25-dihydroxy-vitamin D_3" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, Kentucky.

DR. MICHAEL BINDER (1/1/83 - 12/30/83) worked on "Congenital Heart Malformations and Situs Inversus" in Dr. W.M. Layton, Jr.'s laboratory at Dartmouth Medical School. He is now on a research fellowship in the Pathology Department at Brown University, Providence, Rhode Island.

DR. THOMAS BJÖRKMAN (10/1/86 - 9/30/88) worked on "The Mechanism of Gravity Sensing in Plants" in Dr. Robert Cleland's laboratory at The University of Washington, Seattle, Washington. He is continuing to work in Dr. Cleland's laboratory at the University of Washington, Seattle, Washington.

DR. STEVEN BLACK (7/1/82 - 6/30/84) worked on "Determination by Gravitational and Centrifugal Force of the Amphibian Dorsal-ventral Axis" in Dr. Raymond Keller's laboratory at the University of California, Berkeley. He is continuing research with Dr. Keller and is also working with Dr. Kenneth Souza at NASA-Ames Research Center, Moffett Field, California.

DR. HARRY BLAIR (7/1/84 - 6/30/86) worked on "Cellular Mechanisms of Bone Degradation" in Dr. Steven Teitelbaum's laboratory at The Jewish Hospital/Washington University Medical Center, St. Louis, Missouri. He is continuing to work in Dr. Teitelbaum's laboratory funded by a NIH Physician Scientist Training Grant.

DR. THOMAS BROCK (8/1/86 - 7/30/88) is working on "Comparison of Changes in Protein Synthesis Induced by Gravity and Auxin Treatment in Pulvini and Coleoptiles of Oat (*Avena sativa* L.) in Dr. Peter Kaufman's laboratory at The University of Michigan, Ann Arbor, Michigan.

DR. JAY BUCKEY, JR. (7/1/82 - 6/30/84) worked on "2-D Echocardiography as an Accurate Mean for Measuring Left Ventricular Volume and Central Venous Pressure During Zero-gravity" in Dr. C. Gunnar Blomqvist's laboratory at the University of Texas Health Sciences Center, Dallas. At the present time he is the project manager for the cardiovascular experiment scheduled on Spacelab-4 and a Research Assistant Professor/Instructor in Clinical Medicine at the University of Texas Health Sciences Center, Dallas, Texas.

DR. GEORGE H. BURROWS (7/1/81 - 6/30/83) worked on "Studies of Synaptogenesis" in Dr. Marshall Nirenberg's laboratory at NIH, Bethesda, Maryland. He is now on the staff of the National Heart, Lung, and Blood Institute, Bethesda, Maryland.

DR. DENIS CLOHISY (7/1/86 - 6/30/87) worked on "Mechanisms of Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at The Jewish Hospital/Washington University Medical Center, St. Louis, Missouri. He is now completing his clinical training in Orthopaedic Surgery at the University of Minnesota, St. Paul, Minnesota.

DR. MARK COOPER (1/1/85 - 12/30/86) worked on "Osteoporosis of Weightlessness and the Electrophysiology of Bone" in Dr. John Miller's laboratory at The University of California at Berkeley, California. He is now a Research Associate in the Department of Molecular Neurobiology at Yale Medical School, New Haven, Connecticut.

DR. MARK DESROSIERS (7/1/86 - 6/30/88) worked on "A Search for Voltage-gating of Plant Hormone Transport Channels" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, Michigan. He is continuing to work in Dr. Bandurski's laboratory at Michigan State University.

DR. J. DAVID DICKMAN (6/1/87 - 5/30/89) is working on "High Frequency Response Properties of Semicircular Canal Fibers" in Dr. Manning Correia's laboratory at the University of Texas, Galveston, Texas.

DR. JOHN S. GARAVELLI (1/1/82 - 4/30/82) worked on "Chemical Characterization of Volatile Products of Algal Cell Cultures" in Dr. Franklin Fong's laboratory at Texas A&M University. He is now working for the Extraterrestrial Research Division at NASA-Ames Research Center, Moffett Field, California.

DR. JOHN GAYNOR (1/1/81 - 12/30/82) worked on "Purification and Characterization of Amyloplasts from *Pisum sativum*" in Dr. Arthur Galston's laboratory at Yale University. He is now an Assistant Professor and Henry Rutgers Scholar in The Botany Department at Rutgers University, Newark, New Jersey.

DR. STEVEN GLOTZBACH (1/1/84 - 12/30/84) worked on "Neurophysiological Studies of Circadian Rhythm Control Mechanisms" with Dr. H. Craig Heller at Stanford University and Dr. Charles A. Fuller at the University of California, Riverside. He is continuing to work in Dr. Heller's laboratory funded by a NIH-NIRA grant, Palo Alto, California.

DR. CHERYL GOULD (7/1/84 - 8/30/85) worked on "Effect of Weightlessness on Various Immunological Functions Using a Murine Simulated Space Flight Model" in Dr. Gerald Sonnenfeld's laboratory at The University of Louisville, Louisville, Kentucky. She is now an Assistant Professor at the University of Kentucky, Lexington, Kentucky.

DR. MARTHA GRAY (7/1/86 - 6/30/87) worked on "The Correlation of Applied Strain Distributions to the Location of New Bone Formation: A Rigorous Mechanical Analysis of an *In vivo* Bone Preparation" in Dr. Clinton Rubin's laboratory at Tufts University School of Veterinary Medicine, North Grafton, Massachusetts. She is now an Assistant Professor at the Massachusetts Institute of Technology, Boston, Massachusetts.

DR. EDWARD GREENFIELD (7/1/88 - 6/30/89) is working on "Regulations of Osteoclastic Bone Resorption by Osteoblasts" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, Missouri.

DR. MARCIA HARRISON (7/1/83 - 8/30/85) worked on "Participation of Ethylene in Two Modes of Gravitropism of Shoots" with Dr. Barbara Pickard at Washington University, St. Louis, Missouri. She is now an Assistant Professor in the Biology Department at Marshall University in Huntington, West Virginia.

DR. GARY JAHNS (1/1/83 - 4/30/84) worked on "Interactions of Light and Gravity on the Growth, Orientation, and Lignin Biosynthesis in Mung Beans" in Dr. Joe Cowles' laboratory at the University of Houston. He is now working at NASA-Ames Research Center, Moffett Field, California.

DR. TIMOTHY JONES (1/1/81 - 12/30/82) worked on "The Effects of Hypergravic Fields on Brainstem Auditory-evoked Potentials" in Dr. John Horowitz' laboratory at the University of California, Davis. He is now an Associate Professor at the University of Nebraska, Lincoln, Nebraska.

DR. THOMAS KERR (1/1/83 - 12/30/84) worked on "Cellular Localization of Na^+, K^+-ATPase in the Mammalian Vestibular System"; the first year in Dr. Muriel Ross's laboratory at the University of Michigan and the second year in Dr. Dennis Drescher's laboratory at Wayne State University. He is now an Assistant Professor at Wayne State University, Detroit, Michigan.

DR. DOUGLAS KLIGMAN (7/1/82 - 6/30/84) worked on "The Role of Neurite Extension Factor Nerve and Muscle Tissue Response to Stress or Injury" in Dr. David Jacobowitz' laboratory at the National Institute of Mental Health, Bethesda, Maryland. He is now on the staff at NIMH, Bethesda, Maryland.

DR. KONRAD KUZMANOFF (7/1/83 - 7/30/85) worked on "Isolation and Identification of B-glucan Synthetase: A Potential Biochemical Regulator of Gravistimulated Differential Cell Wall Loosening" in Dr. Peter Ray's laboratory at Stanford University. He is now a Research Associate working with Dr. Craig Beattie at the University of Illinois at Chicago, Illinois.

DR. MICHAEL MATILSKY (1/1/81 - 12/30/82) worked on "Gravity Perception in the Algal Coenocyte *Caulerpa prolifera* " in Dr. William Jacobs' laboratory at Princeton University. He is now a Senior Research Scientist with Plant Biotech Industries in Ashrat, Israel.

DR. KENNETH MCLEOD (11/1/87 - 10/30/89) is working on "*In-vivo* Measurement of Strain Generated Potentials in Bone During Controlled Mechanical Loading" in Dr. Clinton Rubin's laboratory at the State University of New York, Stony Brook, New York.

DR. DEWEY MEYERS (7/1/81 - 6/30/83) worked on "Response, Adaptation and Gravitational Perception in a Parthenogenic Freshwater Microcrustacean, *Daphnia galeata mendotae*" in Dr. Allan Brown's laboratory at the University of Pennsylvania. He was the Science and Curriculum Coordinator in the Space Life Sciences Training Program at Kennedy Space Center, Florida. Recently he became an Adjunct Associate Professor at West Virginia School of Osteopathic Medicine, Lewisburg, West Virginia.

DR. LLOYD MINOR (7/1/87 - 6/30/88) worked on "Primary Vestibular Afferent Inputs to Central Pathways Mediating the Vestibulo-ocular Reflex" in Dr. Jay Goldberg's laboratory at the University of Chicago, Chicago, Illinois. He is now finishing his clinical training at the University of Chicago Medical Center, Chicago, Illinois.

DR. DEAN MURAKAMI (1/1/85 - 12/30/86) worked on "Influences of the Hyperdynamic Environment on the Development of the Visual System in the Rat" in Dr. Charles Fuller's laboratory at the University of California at Davis. He is continuing to work with Dr. Fuller at the University of California, Davis.

DR. MARY MUSGRAVE (6/1/86 - 10/30/88) worked on "Studies of Respiratory Metabolism" in Dr. Boyd Strain's laboratory at Duke University, Durham, North Carolina. She is now an Assistant Professor at Louisiana State University, Baton Rouge, Louisiana.

DR. SHERRIE LYNN PERKINS (7/1/88 - 4/30/89) worked on "Vitamin D Effect on Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, Missouri. She is now funded by a 3-year NIH grant to continue working in Dr. Teitelbaum's laboratory at Washington University.

DR. KENNETH POTE (6/1/88 - 5/30/89) is working on "An Otoconial Calcium Binding Protein; Its Temporal Expression and Tissue Distribution" in Dr. Robert Kretsinger's laboratory at the University of Virginia, Charlottesville, Virginia.

DR. GARY RADICE (7/1/81 - 6/30/83) worked on "Control of Gravity-sensing Mechanism in Amphibian Eggs" in Dr. George Malacinski's laboratory at Indiana University. He is continuing to work with Dr. Malacinski, Bloomington, Indiana.

DR. DENNIS REINECKE (11/1/88 - 3/30/89) worked on "Does Indole-3-Acetic Acid Turnover Correlate with Topically-induced Asymetric Growth?" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, Michigan. He is now an Assistant Professor at the University of Minnesota, St. Paul, Minnesota.

DR. FARREL R. ROBINSON, JR. (7/1/84 - 6/30/86) worked on "Sensory Motor Properties of the Uvula and Nodulus" in Dr. David Tomko's laboratory at the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. He is now working as a Research Associate with Dr. Albert Fuchs in the Physiology Department of the University of Washington School of Medicine in Seattle, Washington.

DR. BRUCE SERLIN (7/1/84 - 6/30/85) worked on "Differential Wall Growth in Gravistimulated Corn Roots: Its Timing and Regulation" in Dr. Stanley Roux's laboratory at the University of Texas at Austin. He is now an Assistant Professor at DePauw University, Greencastle, Indiana.

DR. ROBERT SLOCUM (1/1/81 - 12/30/83) worked on "Studies on the Localization and Functional Role of Calcium in Gravistimulated Plant Organs;" the first year in Dr. Stanley Roux's laboratory at the University of Texas at Austin and the second year in Dr. Arthur Galston's laboratory at Yale University. He is now an Assistant Professor at Williams College, Williamstown, Massachusetts.

DR. J. HENRY SLONE (7/1/85 - 6/30/87) worked on "Characterization of the Protein Responsible for the Lateral Transport of Auxin During Gravitropism of Pea Shoots and Determination Whether Phosphorylation Participates in Gravitropic Activation" in Dr. Barbara Pickard's laboratory at Washington University in St. Louis, Missouri. He is continuing to work with Dr. Pickard in St. Louis, Missouri.

DR. JOSEPH STEFFEN (7/1/81 - 6/30/83) worked on "Glucocorticoid Receptor Levels in Hindlimb Skeletal Muscles and Diaphragm During Prolonged (2 Week) Antiorthostatic Hypokinesia and Recovery" in Dr. X.J. Musacchia's laboratory at the

University of Louisville. He is now an Assistant Professor at the University of Louisville, Louisville, Kentucky.

DR. JULIANNA SZILAGYI (7/1/81 - 12/30/81) worked on "Progressive Hemodynamic Changes in Simulated Weightlessness" in Dr. Carlos Ferrario's laboratory at the Cleveland Clinic. She is now an Assistant Professor at the University of Houston, Houston, Texas.

DR. YASUHIRO TORIGOE (1/1/84) - 12/30/85) is working on "Anatomical Correlated Underlying Vestibulo-autonomic Outflow to the Gut" with Dr. Robert H.I. Blanks at the University of California, Irvine. He is continuing to work with Dr. Blanks at the University of California, Irvine, California.

THE INTERACTION OF SKELETAL REMODELING WITH SYSTEMIC DISORDERS: AN OBSTACLE TO EXTENDED SPACEFLIGHT

Steven D. Bain
Musculo-Skeletal Research Laboratory
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Stony Brook, NY 11794

Description of Research

The objectives and long-term goals of this research are intended to determine how an organism's metabolic status interacts with bone tissue to modulate the ability of the skeleton to remodel in response to changes in its mechanical environment. As participation in space exploration expands to include individuals of increasingly diverse metabolic status, the question of how microgravity will interact with each individual's physiological milieu becomes critical. For example, considering the differences in endocrine driven remodeling between gender, the skeletal changes in female astronauts may differ significantly, and be potentially far more deleterious, than those stimulated in their male counterparts. Indeed, we have proposed that the systemic state of an organism will not only influence, but perhaps control, the nature and extent of skeletal remodeling in response to microgravity conditions. Ultimately, a means to predict the potential skeletal risk of each astronaut candidate could play a pivotal role in the future selection of potential space travelers by identifying those in greatest danger of skeletal distress.

Determining the potential impact of a "distressed" metabolic state on the skeleton's response to disuse requires the capacity to enhance or exclude certain components of the bone's mechanical environment, as well as the ability to control specific aspects of the animal's systemic milieu. These experimental criteria can be satisfied by utilizing an animal model of disuse osteoporosis, the functionally isolated turkey ulna. In this model, while the bone is completely deprived of any mechanical stimuli, its vasculature and innervation are preserved. In addition, through changes in diet (calcium deficiency) or hormonal balance (endocrinopathy), the skeletal response to the superimposed effects of systemic factors can be quantified.

We are using the ulna model to address how the bone responds to the absence of mechanical loading in each of the following systemic populations:

(1) Adult normal (healthy males, 1 yr old, fused physes);
(2) Nutritionally deficient (calcium-poor diet);
(3) Hormonally imbalanced (castrated adult males);
(4) Growing normal males (5 mo of age); and
(5) Old normal males (3 yr of age).

By compiling a detailed morphologic, cellular, and physical profile of the skeleton's response to disuse in healthy adult, endocrinopathized, nutritionally deficient, growing, and aged populations, we are developing a framework with which to evaluate the impact of metabolic status on the bone's ability to adapt to an altered mechanical environment.

Accomplishments

(1) ***Eight weeks of functional isolation of the ulna in normal adult males results in a 15% decrease of bone mass. This bone loss is a result of cortical thinning by the expansion of the marrow cavity.***

(2) ***In castrated males, the same 8 week period of functional isolation generates a decrease in mass identical to that observed in the disuse ulnae of normal males. However, the bone is removed intracortically, not by expansion of the marrow cavity.*** Thus, the ability of the bone cells to perceive and/or respond to changes in the mechanical environment are modulated by the animal's systemic state.

(3) Comparisons of the intact ulnae of castrated and normal males shows an increase in porotic area and the number of intracortical remodeling events in the castrated animals. This suggests that the cortical bone is "pre-activated" by castration, independent of disuse. Therefore, when functional isolation is superimposed on this "primed" system, cortical bone remodeling dominates over endosteal resorption in the development of osteopenia.

(4) ***In animals systemically primed for growth (i.e., 5 mo old males), exposure of the ulnae to disuse does not trigger a decrease in bone mass.*** In fact, functional isolation actually appears to accelerate bone formation rates. Compared to intact control bones, periosteal bone apposition was increased 64% in the disuse ulnae of growing animals. These results suggest that in the young, rapidly growing skeleton, functional stimuli exert a strong controlling influence on bone forming and resorbing activities. Once this control is removed, the active cells escape the regulatory influence of function, and in this case, increase their bone forming and resorbing actions.

Significance of the Accomplishments

It is apparent from our preliminary results that the morphologic and cellular aspects of remodeling are greatly influenced, if not dominated, by the animal's systemic state. It is clear, from the site-specific resorption in the castrated animals to the unaffected girth of the growing animals, that the systemic state must be considered in evaluating the skeletal response to disuse. By evaluating the impact of hormonal imbalance, growth, nutritional deficiency, and aging as regulatory factors in the skeleton's response to disuse, we can assemble a profile of the remodeling interaction potentiated by each metabolic condition. Indeed, if there are distinct mechanisms by which each systemic state interacts with the normal adaptive process, then it should not be surprising to expect a unique morphologic response for each metabolic population.

Spaceflight is becoming accessible to an increasingly diverse population. This will no doubt include scientists chosen not for their physical fortitude, but for their expertise across a wide spectra of disciplines. The metabolic profiles of these payload specialists will be equally as diverse. To minimize the potential trauma to the skeletal system, investigations must be designed to evaluate the influence of age, nutrition, and endocrine related factors on the bone's response to microgravity.

Publications

Bain, S. and Rubin, C. Caponization increases site specific bone loss in the functionally isolated avian ulna (Abstract). *Journal of Bone and Mineral Research* 3(Suppl.): S190, 1988.

Bain, S.D. and Rubin, C.T. Priming the osteopenic response: Modulation of disuse osteoporosis by testosterone deficiency (Abstract). *Transactions of the 35th Orthopaedic Research Society,* 14: 16, 1989.

Bain, S.D. and Rubin, C.T. Site specific bone remodeling in the functionally isolated ulnae of castrated male turkeys (Abstract). *ASGSB Bulletin* 2: 42, 1989.

IMMUNE CHANGES DURING UNLOADING MODELS OF MICROGRAVITY: RELATIONSHIP TO DIHYDROXYVITAMIN D

Wallace D. Berry
Department of Microbiology and Immunology
University of Louisville School of Medicine
Louisville, KY 40292

Description of Research

The long-range objective of this research is to define the mechanisms responsible for immune system changes during exposure to microgravity. Our present goal is to determine how changes in the concentration of circulating 1,25-dihydroxyvitamin D ($1,25(OH)_2$ D) affect components of cellular immunity during unloading simulations of microgravity exposure.

Functional changes in components of cellular immunity accompany adaptation to the spaceflight environment. Lymphocytes from humans and animals flown in space show reduced proliferative responses to mitogenic stimulation and may have reduced ability to produce cytokines. The physiological mechanisms responsible for these immunological effects of spaceflight are unknown.

Recent discovery of the immunoregulatory effects of vitamin D suggest that bone metabolism and mineral homeostasis, which are altered during spaceflight, may interact with the immune system through the vitamin D hormone, $1,25(OH)_2$ D. The main points of our hypothesis are: (1) *microgravity induced changes in bone and mineral metabolism decrease circulating concentrations of $1,25(OH)_2$ D, and (2) subnormal levels of $1,25(OH)_2$ D reduce immune cell activity and production of cytokines* (Figure 1).

We are using a rat unloading model to test this hypothesis. Varying periods of whole body unloading, with and without head down tilt, are being used to reproduce the musculoskeletal unloading and headward fluid shifts characteristic in microgravity. Plasma levels of $1,25(OH)_2$ D are measured following each unloading period. Lung macrophages from the unloaded rats are tested for changes in their ability to phagocytize sheep red blood cells. Spleen cells (monocytes and lymphocytes) are assayed for their ability to produce the immune cytokines interleukin-1 (IL-1), interleukin-2 (IL-2), interferon-gamma (IFN-gamma), and interferon-alpha/beta (IFN-alpha/beta). These components of cellular immunity are also being examined in rats reared on a vitamin D deficient diet. This allows us to compare the immunological effects of unloading with those of dietary vitamin D deficiency.

Accomplishments

(1) ***We have confirmed that unloading models significantly reduce the concentration of $1,25(OH)_2$ D in the blood.*** Circulating $1,25(OH)_2$ D was reduced by more than 60% following seven days of head down unloading and by more than 70% following fourteen days of head down unloading. ***Unloading without head down tilt decreased plasma $1,25(OH)_2$D exactly as in head down unloading.***

(2) Unloading with or without head down tilt for seven or fourteen days had variable effects on the ability of macrophages to phagocytize foreign particles. Some experiments have shown significantly reduced phagocytosis while others have shown slightly increased phagocytosis. Macrophage phagocytic ability appeared to be reduced in rats on a vitamin D deficient diet.

HYPOTHETICAL MECHANISM FOR MICROGRAVITY EFFECTS ON RAT IMMUNE SYSTEM

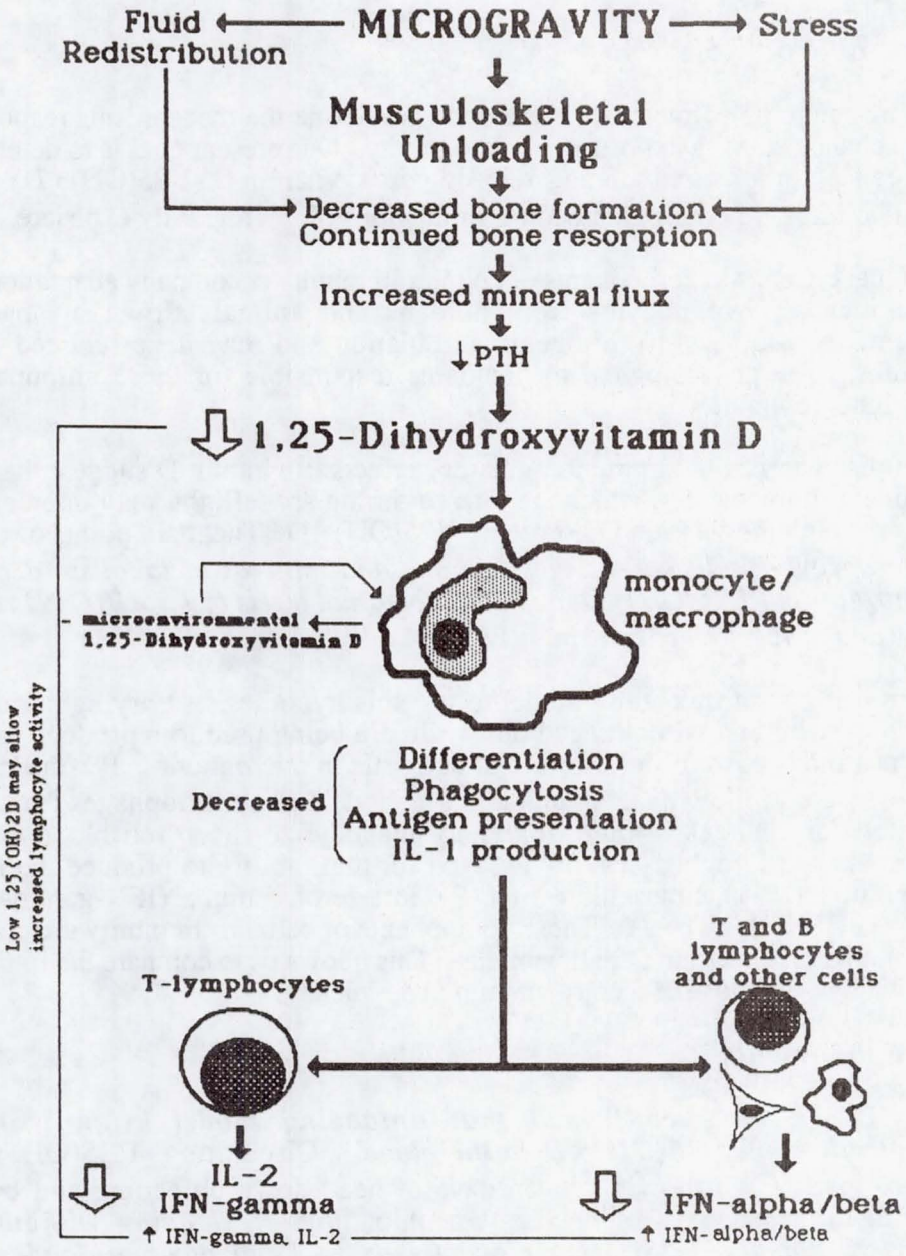

Figure 1. Possible mechanism for microgravity induced changes in the immune system mediated by 1,25-dihydroxyvitamin D.

(3) ***Spleen cell production of IL-1 was reduced by seven or fourteen days of unloading with or without head down tilt. Spleen cells from rats fed on the vitamin D deficient diet had similar reductions in their IL-1 production.***

(4) Spleen cell production of IL-2 was inconsistently affected by unloading. Reduced IL-2 production was seen following seven days of unloading with or without head down tilt in some experiments, while enhanced production was seen after fourteen days of unloading. Dietary vitamin D deficiency did not affect IL-2 production.

(5) ***Reduced IFN-gamma production was seen following seven or fourteen days of unloading regardless of tilt.*** The effects of dietary vitamin D deficiency are currently being assessed.

(6) Unloading models had variable effects on production of IFN-alpha/beta. Some experiments have shown as much as 70% suppression of IFN-alpha/beta production following unloading while other experiments have shown enhancement of production.

Significance of the Accomplishments

Finding #1. These studies confirm that unloading models profoundly affect vitamin D metabolism and that fluid shifts or associated factors do not contribute to the observed decrease in the plasma $1,25(OH)_2$ D of unloaded rats. These findings raise the possibility that a similar decrease in $1,25(OH)_2$ D will occur during spaceflight or other forms of skeletal unloading such as chronic bedrest or paralysis. These findings are also significant in light of evidence that $1,25(OH)_2$ D is required for normal function of the immune system, brain, vestibular system, pancreas, pituitary, bone, and other organs.

Findings #2-6. Interleukins and interferons are cytokines that function as immune system hormones and as antiinfective agents. Phagocytosis and IL-1 production by monocytes/macrophages are central to the initiation of immune responses and recruitment of other components of cellular immunity. Loss of phagocytosis and IL-1 production can inhibit lymphocyte activity and production of cytokines such as IL-2 and interferons. Interleukin-2 is important in sustaining and reinforcing cellular immune responses and in differentiation of immune cells. The variability of IL-2 production in lymphocytes of unloaded rats may be due to competition between the positive effects of decreased circulating $1,25(OH)_2$ D and the negative effects of low IL-1. Interferon-gamma regulates the appearance of molecules that allow self-nonself discrimination during immune responses, stimulates macrophage activity, and promotes IL-1 production. Also, IFN-gamma may be synergistic with $1,25(OH)_2$ D to promote macrophage killing of microorganisms. Both IFN-gamma and IFN-alpha/beta have significant roles in cell differentiation and as antiviral agents. The expected result of impaired ability to produce these cytokines would be altered immune cell subpopulations, decreased resistance to infection, and/or inappropriate immune responses.

In summary, our findings indicate that unloading models cause specific changes in immune function in coordination with decreased circulating $1,25(OH)_2$ D. This modeling has selective immunological effects rather than an overall immunosuppression. The immunological effects of unloading also appear to be consistent with changes that occur during vitamin D deficiency. The results of our studies also indicate that these conditions of skeletal unloading and stress, rather than factors associated with head down tilt, are responsible for the vitamin D and immune system effects.

Publications

Berry, W.D., Murphy, J.D., Taylor, G.R., and Sonnenfeld, G. Calcitriol, macrophage function, and cytokine production in a suspension model of microgravity (Abstract). *FASEB Journal* 3: A1367, 1989.

Berry, W.D., Murphy, J.D., Taylor, G.R., and Sonnenfeld, G. Effects of antiorthostatic, hypodynamic, hypokinetic, suspension on interferon production and levels of 1,25-dihydroxyvitamin D_3 in rats (Abstract). *Journal of Interferon Research* 8: S154, 1988.

Berry, W.D., Murphy, J.D., Taylor, G.R., and Sonnenfeld, G. Effects of suspension modeling on macrophage function, lymphokine production and dihydroxyvitamin D (Abstract). *ASGSB Bulletin* 2: 42, 1989.

THE MECHANISM OF GRAVITY SENSING IN PLANTS

Thomas Björkman
Department of Botany, KB-15
University of Washington
Seattle, WA 98195

Description of Research

This research is intended to identify events associated with the initial sensing of gravity and the differential signal produced by the sensing cells.

Whereas the signal from the maize root cap to the root body moves extracellularly, a change in its activity should be measurable in the apoplasm. To test the hypothesis that calcium is that signal, I am directly measuring the calcium activity in the root tip.

For the purpose of identifying the nature of the mechanoreceptor with which statoliths interact, I have analyzed the energetics of receptor activation. This analysis considers the energy available for activation, the observed behavior of statoliths, and the observed sensitivity and speed of gravity perception.

Accomplishments

(1) Corn root tips have a calcium activity (measured with a calcium-specific microelectrode) of 2-6 mM when grown on germination paper wetted with buffer having an activity of 0.2 mM. The activity is the same in intact roots and in roots that have been decapped and grown for up to 24 hr. The calcium activity is restored within 5 min after washing with 1 mM EGTA or with 10 mM $CaCl_2$.

(2) ***Preliminary data suggest a small decline in the calcium activity on the upper side of the sensing region of gravistimulated corn roots.*** More data are needed to say whether the change in calcium activity corresponds to the differential calcium flux measured by Lee and Evans, and whether it is large enough to have physiological consequences.

(3) ***The activation energy of the gravity receptor, whatever its chemical and physical basis, must be close to 4 x 10^{-20} J*** (Figure 1). (This is approximately the energy released from the hydrolysis of one molecule of ATP.) If the activation energy were less, the receptor would be continuously activated and unable to respond to a gravity stimulus. If the activation energy were higher, the effect of gravity would not be energetic enough to activate the receptor. ***If the receptor is a mechanically regulated channel activated by amyloplast sedimentation, the minimum sedimentation would be about 1 μm, and would take 1 to 50 seconds at observed sedimentation rates. These estimates correspond closely to what has been observed.***

Significance of the Accomplishments

The calcium activity in the apoplasm of seedling corn roots is high and well-buffered. The calcium diffusion gradient is out of the root, indicating that calcium is diffusing from the seedling into the surrounding medium. The high and stable calcium concentration implies that a biogenic calcium gradient would be strongly attenuated. Nevertheless, a change in

calcium concentration has been tentatively identified following gravistimulation, implying that the cells have a large capacity for modulating extracellular calcium activity.

The determination of the approximate activation energy is useful because proposed gravity receptors can be discounted if their predicted activation energy is much different from this estimate. Further, it is certain that the velocity of sedimentation cannot be detected; rather, it is the displacement of a statolith which does the work to activate a receptor.

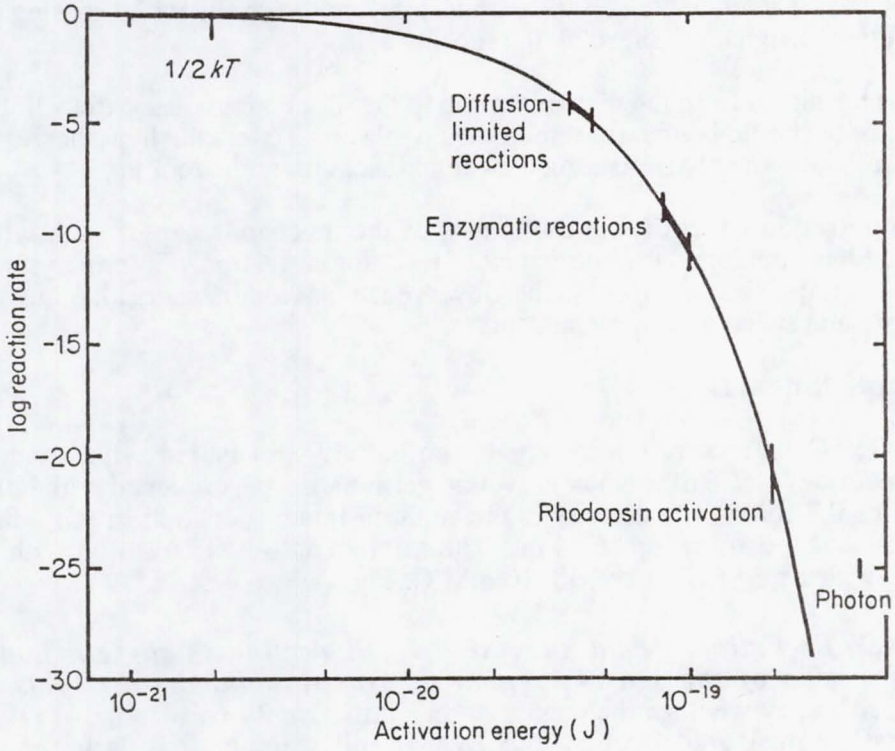

Figure 1. The effect of activation energy on the relative rate of reaction. Enzymic reactions have activation energies clustered over a small range, yet one where the rate of a reaction can vary by many orders of magnitude. Spontaneous thermal activation of the light-sensing molecule rhodopsin occurs at a very low rate. A gravity sensor must be insensitive to thermal energy ($1/2kT$), so its activation energy would be expected to be 3-4 x 10^{-20} J. Intermediate marks on the abscissa are 2 and 5 times the order of magnitude. The formula for this curve is: $\log R = -E_A/kT$. (From *Advances in Botanical Research*, 1988.)

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VOLTAGE GATING OF PLANT HORMONE TRANSPORT CHANNELS

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Description of Research

The objective of this research is to understand the vectorial growth response of plants to gravity. Our working hypothesis is that the plant's internal bioelectrical gradients (currents) serve as sensors and transducers of the gravity response of plants. Changes in a plant's orientation with respect to gravity are followed by a realignment of the plant's internal bioelectric fields, and this shift in the internal bioelectric fields opens and/or closes voltage-sensitive hormone (indole-3-acetic acid, IAA), transporting channels between the plant's vascular tissue (stele) and the surrounding cortical tissue (cortex), leading to an accumulation of the hormone on the lower side of the shoot. This asymmetric hormone distribution in the shoot results in lateral asymmetric growth until the plant's major axis is again vertical.

We previously tested this hypothesis by studying ways in which the application of an external electric potential to young corn shoots alters their growth, the concentrations of the growth hormone IAA in the growing tissues, and the transport of growth-supporting material up the shoot (calcium, IAA, and glucose). In the past year we concentrated on the role that the voltage sensitive channels in the stele:cortex junction play in the transduction processes by examining the transport of apoplastic dyes and measuring the electrical impedance across the stele:cortex junction.

Accomplishments

(1) The growth rate of corn shoots subjected to low electric potentials (0.6 V/cm) was dependent only on the polarity of the applied potential. The growth rate of shoots decreased 90% when the tip of the shoot was held electrically positive, whereas the reverse polarity had no effect on the growth rate. This was mirrored in the effect the applied potential had on the endogenous IAA concentrations in the tissues and in the transport of calcium from the seed to the actively growing tissues, but not in the transport of IAA from the seed.

(2) ***The radial impedance from the interior cortex surface (stele removed) to the outer surface of the cortex was lower in the region of the shoot where active growth occurs,*** compared to the rest of the shoot (Figure 1).

(3) ***An apoplastic dye, light green, would not penetrate the stele:cortex barrier in response to externally applied potentials of either polarity.***

Significance of the Accomplishments

Finding #1: This implied that the control mechanism regulating electrically modified growth resides in the junction between the stele and the cortex tissues.

Finding #2: The reduced impedance in the active growth region suggests more open channels between the stele and cortex tissues in the active growing region of the shoot than in the rest of the shoot.

Finding #3: The communication across the stele:cortex interface is not through the apoplast but through the symplast of the plant. The apoplastic communication between the stele and the cortical tissues is not voltage controlled.

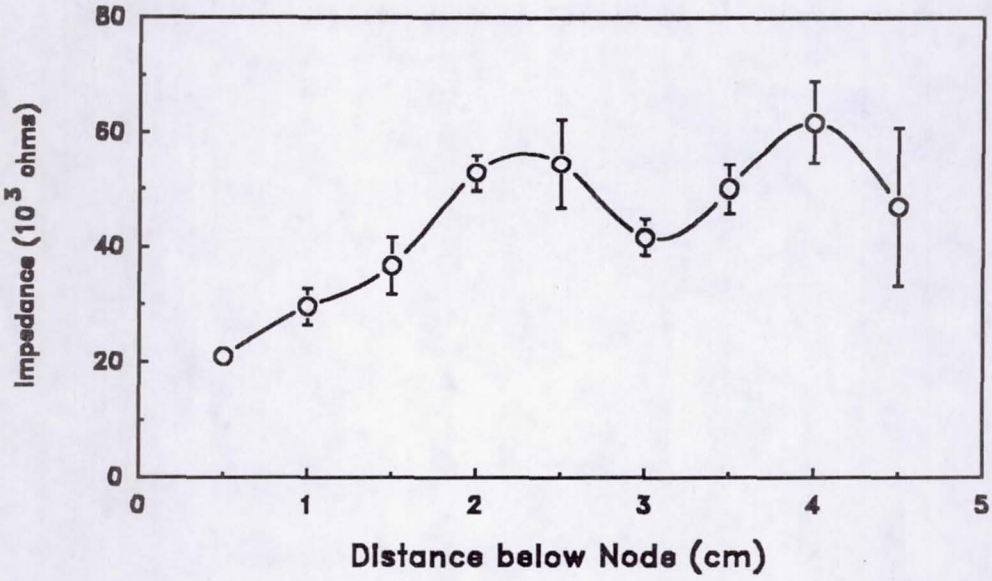

Figure 1. The radial impedance of the mesocotyl cortex. Error bars are standard error of mean.

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SEMICIRCULAR CANAL AFFERENT RESPONSES TO HIGH FREQUENCY STIMULATION

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Description of Research

The natural stimulus of the vestibular system is motion, and most investigators have utilized rotational stimulation in order to study the dynamic responses of semicircular canal afferents (SCA). However, because of technical restrictions such as rotator performance or inability to maintain fiber isolation during high frequency motion, most of these studies have been limited to frequencies of oscillation below 16 Hz. Recently, we have developed a mechanical stimulation technique in order to study the responsiveness of semicircular canal afferents to high amplitude and high frequency stimulation (Dickman, Reder, & Correia, 1988). During the past year, we have utilized the mechanical stimulation technique in order to delineate the response dynamics of pigeon SCA fibers throughout their response bandwidth.

In these experiments, recordings from isolated semicircular canal afferent fibers were obtained in anesthetized pigeons (*Columba livia)* using standard electrophysiological techniques. The semicircular duct of the horizontal canal was exposed for mechanical and rotational stimulation. Horizontal canal afferent (HCA) fiber responses were recorded while delivering independent mechanical or rotational sinusoidal stimuli across a frequency bandwidth ranging from 0.01 to 200 Hz. The magnitude and phase of the response to each stimulus was quantified and then compared as to type of stimulation (i.e., mechanical or rotational) and frequency. Transfer functions were then fit to the frequency response data.

Accomplishments

Mechanical vs. Rotational Stimulation

(1) Responses from HCA fibers to mechanical stimulation were shown to be sensitive, discrete, and reproducible. Stimulation of one semicircular duct (e.g., the horizontal) was shown to elicit responses only from fibers that innervate that canal and not from afferent fibers innervating the other vestibular organs.

(2) When both mechanical and rotational stimulation were applied to the same HCA fiber at various stimulus intensities, comparable responses were obtained. The determined constant of proportionality found to equate the peak response produced by rotational to that elicited by mechanical stimulation was 7.0 $deg.sec^{-1}/1.0\ \mu m$.

(3) Bode plots and best fit transfer functions of the frequency response data ranging from 0.05 to 10 Hz for HCA fibers exposed to both mechanical and rotational stimulation were nearly identical. Parameters for the best fit transfer functions were in excellent agreement with previous rotational studies values reported for the pigeon.

High Frequency Responses

(1) Sinusoidal mechanical stimulation was used exclusively to study the response properties of HCA fibers to stimulus frequencies above 10 Hz. Mechanical stimulation produced clearly entrained action potentials in HCA fibers up to a frequency of 400 Hz (highest stimulus frequency tested), with stimulus probe displacements ± 1.0 and $\pm 2.5\ \mu m$.

(2) The number of action potentials per stimulus cycle decreased as stimulus frequency increased, until only one action potential per stimulus cycle was elicited. The point at which only one spike per stimulus cycle was observed was dependent upon both the fiber's resting mean discharge rate and the fiber's coefficient of variation (a measure of spike discharge regularity over time).

(3) The dynamic response properties of individual HCA fibers were found to be correlated with the fiber's coefficient of variation and the resting level mean discharge rate. Bode plots of the frequency response data for HCA fibers indicated that the upper corner frequency for these afferents was approximately 40 Hz for regular firing HCA fibers and approximately 80 Hz for the more irregular firing HCA fibers.

Significance of the Accomplishments

In the present series of studies, we have demonstrated that ***HCA fiber responses to mechanical and rotational stimulation can be directly equated. The response dynamics produced by these two methods of stimulation are practically identical over the bandwidth ranging from 0.05 to 10 Hz.*** With high frequency mechanical stimulation, the response properties of HCA fibers were particularly interesting. For most HCA fibers, the response bandwidth was determined and the upper corner frequency was dependent upon the individual fiber's mean resting level discharge and coefficient of variation. It has been previously stated that the vestibular system's dynamic response range would develop according to the natural environmental requirements for different species' head movements. Thus, low frequency head motions in amphibians or fish as compared to high frequency head motions in birds may translate to different vestibular system response characteristics for these groups of animals. Further, if vestibular adaptation occurs in microgravity in response to new environmental head motion requirements, it is possible that the semicircular canal system dynamics may also change. The mechanical stimulation technique has provided some information regarding the response dynamics of HCA fibers in their natural environment. It should now be possible to extend these studies to address questions regarding altered gravity environments and their influence upon vestibular system dynamics.

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REGULATION OF OSTEOCLASTIC BONE RESORPTION BY OSTEOBLASTS

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Description of Research

Weightlessness causes rapid bone loss that can dangerously weaken the skeleton. Thus, understanding the processes which control the balance between bone degradation by osteoclasts and bone formation by osteoblasts is essential to allow prolonged spaceflight.

Conditions that increase bone resorption, including microgravity exposure, appear to activate osteoclasts indirectly by interacting with intermediary cells, probably osteoblasts. Thus, it has been hypothesized that osteoblasts produce factors that regulate osteoclast activity. Accordingly, this research has focused on identifying osteoblast-derived factors that directly affect resorption by isolated osteoclasts.

Accomplishments

We initially demonstrated that ***medium conditioned by isolated chicken embryo calvaria osteoblasts stimulates the resorptive activity of highly purified chicken osteoclasts.*** The conditioned medium does not itself induce bone degradation at either pH 7.4 or 5.0 and thus contains a factor that directly activates osteoclasts. In contrast, fibroblast-conditioned medium does not contain the stimulatory activity. Thus, ***production of the factor is an osteoblast-specific property.***

Dialysis and ultrafiltration techniques indicate that the molecular weight of the stimulatory factor is greater than 5000. Subsequently, ***high performance liquid chromatography gel filtration (Bio-Rad TSK-125) revealed a molecular weight of >10,000 and provided >100-fold purification of the stimulatory factor.***

Significance of the Accomplishments

The use of isolated osteoclasts as target cells for the osteoblast-conditioned media is a major advance. Previous studies have used intact bones for this purpose and, thus, have been unable to determine to which cell the stimulatory factor targets, but our experiments clearly demonstrate that the stimulatory factor acts directly on osteoclasts. In addition, the isolated osteoclast approach makes it possible to include hundreds of assays in a single experiment. As a result, testing stimulatory activity of large numbers of chromatographic fractions and purification, identification, and characterization of the factor become feasible. In fact, with this approach, we have already purified the factor >100-fold.

Moreover, the finding that the stimulatory activity is not produced by fibroblasts indicates that it is bone-specific. Thus, understanding the regulation of factor production and function may allow therapeutic control of bone resorption without affecting other physiological processes.

Publications

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REGULATION OF BONE REMODELING ACTIVITY THROUGH THE CONTROL OF STRESS GENERATED ELECTRIC FIELDS

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Description of Research

The long-term objective of this research is to improve our understanding of the manner in which bone is able to sense the demands of its functional environment. More immediately, this work should lead to the development of an exercise regimen appropriate for maintaining bone mass during spaceflight. The basis for this work lies in the electrophysiological properties of bone tissue. During normal functional activity, electrical currents are endogenously induced within bone. Moreover, bone cells are extremely sensitive to these electric currents. Electric fields exogenously induced into bone can be shown to modulate bone remodeling activity in the absence of normal functional loading. These observations suggest that an efficient mechanical loading protocol for the minimization or prevention of microgravity induced bone loss might be developed by optimizing the endogenously induced electrical currents.

The specific objective of this investigation is to define a loading paradigm for the maintenance of bone mass in the absence of normal functional loading. Our hypothesis is that a loading paradigm which maximizes the endogenous generation of osteogenic electric fields will also maintain bone mass with a minimal mechanical work input. This work therefore requires both the isolation of the most osteogenic electric field components of functional strain, and the subsequent testing of the predicted mechanical loading paradigm.

The experimental work is based on the isolated avian ulna model of disuse osteopenia. In this model, the ulna of adult male turkeys is isolated by proximal and distal osteotomies such that the animal cannot apply functional loading to its ulna. Through the use of external magnetic coils, electrical fields mimicking those produced endogenously can be induced in the absence of functional loading. This permits the isolation of the fields which are most osteogenic. These field characteristics are then used to predict an appropriate mechanical loading protocol which can be subsequently tested using the isolated ulna preparation.

Accomplishments

(1) Through the application of broad spectrum electromagnetic fields we have isolated the regime of maximum electric field efficacy to the frequency range below 75 Hz.

(2) Using sinusoidal stimulation within this frequency range we have demonstrated that induced electric fields near 15 Hz demonstrate the greatest osteogenic effect.

(3) At 15 Hz we have shown that field intensities on the order of 10 microvolts per centimeter are sufficient to initiate new bone formation.

(4) *In-vitro* stress generated potential recordings indicate that field intensities on the order of 10 microvolts/cm can be induced with peak bone strains on the order of 100 microstrain.

(5) ***We have shown that the application of 15 Hz loads to the isolated ulna preparation at an amplitude sufficient to produce a strain of 500 microstrain will prevent the normal bone loss expected in the disuse preparation and will also induce substantial new bone formation.***

Significance of the Accomplishments

A possible role for the endogenously produced electrical currents in bone and the phenomena of bone adaptation has been suggested by many investigators, but there has been little supporting evidence for a causal relationship. These studies suggest the relationship between endogenously induced electric fields and remodeling activity may well be causal. The electric fields most effective in mediating bone remodeling activity also predict the more effective mechanical loading paradigms. By utilizing a relatively high frequency mechanical loading paradigm developed utilizing the electrical response data, we can demonstrate the initiation of new bone formation by mechanical loads with peak values less than one tenth those normally experienced by the bone. It is expected that the maintenance of bone mass would require even lower strain levels.

This work seems to indicate that bone senses and adapts to the relatively low amplitude but high frequency strains presented within its environment, rather than the much larger amplitude but lower frequency components of bone strain. As the high frequency components arise predominantly from the dynamics of muscle activity, and not through the reaction loads associated with impact, these results suggest that microgravity bone loss may well be preventable without strenuous activity. These results may contribute to the treatment of both postmenopausal and senile osteoporosis.

Publications

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VITAMIN D EFFECTS ON OSTEOCLAST DIFFERENTIATION

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Description of Research

This research is directed towards understanding the effect of vitamin D on proliferation and differentiation of osteoclast precursors from bone marrow macrophage progenitors. Osteoclasts are bone-resorbing cells, and an imbalance of bone resorption and bone formation leads to osteoporosis, a major problem experienced during spaceflight. Vitamin D has been shown to act as a factor which induces differentiation in many cell types, including bone marrow macrophages, which may eventually differentiate to form osteoclasts. Study of the process of osteoclast differentiation as mediated by vitamin D can give insights into the recruitment of cells to form osteoclasts, osteoclast differentiation and, ultimately, the processes of osteoporosis.

This research has focused on early murine bone marrow precursor cells, which may be enriched by pretreating the animal with the chemotherapeutic agent, 5-flurouracil. Collection of the marrow one day after treatment yields an early precursor population which is characterized by its requirements for the growth factors CSF-1 and IL-1 for growth, as well as the presence of low levels of the CSF-1 membrane receptor. Marrow collected 5 days after treatment yields intermediate precursors, which require CSF-1 or IL-3 for growth and have higher levels of the CSF-1 receptor expressed. By utilizing these two populations of cells, the effect of vitamin D on differentiation can be followed as the cells mature.

To study the effects of vitamin D on differentiation, the parameters of cell division and expression of the CSF-1 membrane receptor were studied, as well as morphological expression of macrophage-like characteristics. The cessation of cellular division is associated with differentiation, and the cells begin to show macrophage-like characteristics such as plastic adherence, expression of non-specific esterase activity and increasing expression of the membrane CSF-1 receptor. Cellular proliferation was studied utilizing ^{3}H-thymidine incorporation and a soft agar colony proliferation assay with morphological analysis. Expression of the CSF-1 receptor was followed utilizing cold competing membrane binding assays of both adherent and non-adherent cell populations.

Accomplishments

Studies utilizing murine bone marrow showed that ***vitamin D affects the differentiation of both early and intermediate bone marrow osteoclast precursor cells. Both early and intermediate precursor populations showed a marked inhibition of proliferation as measured by ^{3}H-thymidine incorporation or soft agar colony assays. Additionally, treatment of the early precursor cells with vitamin D caused an earlier increase in expression of the CSF-1 receptor, indicating that the steroid has induced differentiation of these cells. Conversely, treatment of the intermediate cell population caused a decrease in expression of the CSF-1 receptor as***

compared to controls. This may represent a differentiational branch point, as macrophages continue to express the CSF-1 receptor whereas osteoclasts do not.

All of the effects seen above were in cells which developed characteristics of macrophages including plastic adherence and expression of non-specific esterase. Additionally, the vitamin D effects on proliferation and CSF-1 receptor expression were found to be dose-dependent, with an optimal dose of 10^{-8}M (which is physiologic in the concentrations of serum used) and metabolite-specific for 1,25 $(OH)_2$ vitamin D_3.

Significance of the Accomplishments

These findings indicate that vitamin D is acting to promote differentiation of osteoclast precursor cells at multiple stages of development. This suggests that the steroid is an important mediator of osteoclastogenesis. If osteoporosis is viewed as an imbalance of bone resorptive- and bone-forming processes, then the ability to inhibit osteoclastogenesis would favor new bone formation and lessen bone loss. Osteoporosis during long-term weightlessness remains one of the main blockades to extended spaceflight. Thus, the continued study of the processes of osteoclastogenesis as well as the growth and differentiation factors which mediate the recruitment of cells and their eventual formation into osteoclasts will allow insights into mechanisms of osteoporosis and possible prevention of the process.

Publications

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TEMPORAL EXPRESSION AND SPATIAL DISTRIBUTION OF THE MAJOR PROTEINS IN THE OTOCONIA OF TWO SPECIES OF VERTEBRATE

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Description of Research

This research is aimed at increasing our understanding of the function of the peripheral portion of the vestibular system found in the inner ear of vertebrates. Specifically, the research focuses on the evolution, chemistry, and function of a biomineralized structure found within the inner ear. The vestibular system has evolved to detect acceleration and gravity through five sensory specializations in each ear. Three sensory end organs, the cristae ampullarae, are located in dilatations of the semi-circular canals and are sensitive to angular acceleration. The end organs known as the maculae detect linear acceleration through the interaction of extracellular mineralized structures with the underlying neuroepithelium. These biominerals, known as otoconia (literally "ear dust"), are embedded in a gelatinous mass known as the otoconial membrane. The otoconia are thought to increase the mass of the otoconial membrane, thereby increasing the deflection of the apical projections on the underlying sensory cell, the hair cell, during accelerations.

Otoconia are composites of protein and mineral phases. The mineral phase varies within the vertebrates in a phylogenetic trend. Otoconia from some primitive fishes are mineralized by calcium carbonate deposited as vaterite. Reptiles and amphibians predominantly have otoconia mineralized by calcium carbonate in the crystal form of aragonite. The otoconia from birds and mammals contain calcium carbonate in the form of calcite. This research has focused on the proteins contained within the otoconia of the rat (calcitic) and the African clawed frog, *Xenopus laevis*, (aragonitic). Little is known about the nature of these proteins, save their molecular weights. The calcitic rat otoconial protein is M_r = 90k and the aragonitic is M_r = 22k. The cells of origin, developmental expression, or turnover of the proteins are all unknown. In addition, nothing is known about the nature of these proteins' interactions with their mineral component. Understanding the nature of these proteins will enhance our knowledge of the development and evolution of the mammalian vestibular system and will serve as a model system for the field of biomineralization.

I have begun to investigate the otoconial proteins using both immunological/histochemical and molecular biological approaches. My plan is to isolate the proteins from the otoconia of the rat and the clawed frog, raise antibodies against them, and use these antibodies to perform immunohistochemistry studies, and screen cDNA libraries constructed from inner ear tissues. The clones which code for the proteins will be used to generate *in situ* hybridization probes and for construction of expression clones for the production of the proteins in large amounts. Both the cDNA probes and the antibodies are to be used to study the development and evolution of otoconia. These approaches will enable me to determine of the temporal and spatial expression of these proteins. In addition, they will allow the further characterization of these proteins and the nature of their interactions with the mineral phase using *in vitro* approaches.

Accomplishments

In the past year I have raised antibodies against the proteins from both the rat and frog otoconia. The antisera were raised by injection of electrophoretically separated proteins into guinea pigs. These antibodies are of high avidity and specificity, especially in light of their being from polyvalent sera. Both antisera will detect approximately 1 ng of antigen when the sera are diluted at 1:10,000 on a western blot. Further, these sera do not detect any proteins from other tissues of the rat or frog. In addition, the sera do not recognize the other otoconial protein. That is, the antiserum raised against the rat 90k otoconial protein does not cross react with the 22k from otoconial protein. The same specificity is true for the sera raised against the 22k frog otoconial protein. Finally, the antisera do not recognize any other proteins from the inner ears of these two animals. The specificity and avidity of these sera are of adequate quality to be used for immunohistochemistry studies and for screening the cDNA libraries.

Immunofluorescent localization of both the antigens within the inner ear has begun. For the rat I have seen apparent localization in the epithelia underlying the otoconial membrane. This appears to be localized to the hair cell. There is also staining of the decalcified matrix from within the otoconia. In the frog inner ear there appears to be localization within the epithelial cells but I have not seen any localization within a matrix from within the aragonitic otoconia. In fact, it appears that the aragonitic otoconial matrix is not preserved by normal histological preparation.

I have begun investigations using the construction of cDNA libraries from the inner ears of both the rat and the clawed frog. These libraries were constructed in λZAP. This vector is designed for screening with either antibodies or oligonucleotide probes. I am still in the process of screening the libraries for the expression of the otoconial protein. To date no clones containing the cDNA for either otoconial protein have been isolated.

Significance of the Accomplishments

These results are significant primarily for two reasons. First, the antibodies will enable both the screening of the cDNA libraries and the immunohistochemical localization of the antigens within the inner ear of these two vertebrates. Second, the generation of the cDNA libraries will enable a modern approach to the study of the vertebrate inner ear and peripheral vestibular system.

In terms of immediate significance, the antibodies have given results which demonstrate several features of the otoconial proteins. First, ***from the western blots, it has been demonstrated that the otoconial proteins are most likely unique to otoconia.*** For instance, these proteins are not found in the gut, liver or, more importantly, in bone. Second, if the proteins from the calcitic otoconia (those of the rat) evolved from the aragonitic proteins (the frog), they no longer share the same antigenic determinants. Another explanation is that these proteins do not share a common evolutionary ancestor. Lastly, the immunohistology gives ***some evidence that otoconia in the aragonitic form may have a different mode of mineralization than that of calcitic otoconia.*** This matter is still under investigation.

Publications

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DOES INDOLE-3-ACETIC ACID TURNOVER CORRELATE WITH TROPICALLY-INDUCED ASYMMETRIC GROWTH?

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Description of Research

Plant hormones play an important role in terrestrial plant growth and development. In microgravity, dark-grown seeds germinate into long winding plants, while terrestrial germinated plants grow vertically. The plant hormone indole-3-acetic acid (IAA) is rapidly redistributed to the lower faster-growing side of a shoot following a gravity stimulus. The long-range goal of this research is to understand how gravity induces a hormone/IAA asymmetry, and how microgravity may affect development by means of altering hormone metabolism.

This research has examined one aspect of IAA metabolism which may contribute to this rapid hormone asymmetry — catabolism. In corn the first reaction in the catabolism of IAA is oxidation to oxindole-3-acetic acid. This pathway is irreversible, and results in loss of IAA/hormone activity. I have developed the first *in vitro* assay for this enzyme to aid in characterizing this pathway. The regulation of this pathway may help maintain IAA growth-limiting by inactivating the hormone following growth induction. The current project has focused on understanding the mechanism of this pathway.

Accomplishments

Accomplishments include the further characterization of the IAA to oxindole-3-acetic acid pathway: ***separation of oxindole-3-acetic acid oxygenase from peroxidase and lipoxygenase, the first determination of this enzyme's apparent molecular weight, and the demonstration that this pathway occurs in green vegetative tissues.*** The *in vitro* assay depends on a lipid-soluble cofactor which can be substituted by fatty acids. ***The present study gives evidence that IAA oxidation to oxindole-3-acetic acid is by a novel enzyme distinct from peroxidase or lipoxygenase.***

Significance of the Accomplishments

General characteristics of this enzyme have been extended. The enzyme has been shown to be a novel enzyme which can be separated from interfering peroxidase and lipoxygenase enzymes by gel filtration and ion exchange chromatography. In the regulation of the enzyme activity during gravitropism, the amount of enzyme or more likely the amount of the cofactor will be regulated. To isolate, identify, and quantify the cofactor, it is necessary to isolate the IAA oxygenase free of interfering enzymes and use the purified enzyme to screen chromatographic fractions for the active fraction. (It is of interest to determine whether the lipid-soluble cofactor is also co-oxidized during IAA oxidation, and if so, does the co-oxidized product have any biological activity.) Measuring the turnover rate of IAA to oxindole-3-acetic acid and measuring the levels of the cofactor will clarify how catabolism down-regulates IAA activity during environmental changes. The separation away of interfering enzymes and identification of the natural cofactor makes this research feasible.

Previously, decarboxylation of IAA by peroxidase was thought to regulate IAA levels. This work has confirmed that IAA oxidation to oxindole-3-acetic acid occurs without the involvement of peroxidase. Further work has shown that the enzyme occurs in young green seedlings as well as etiolated seedlings. The pathway occurs at two distinct developmental periods and is not only a germination phenomenon.

The basic understanding of how gravity induces a hormone asymmetry, and how microgravity affects normal plant development by altering hormone metabolism and action will likely have practical benefits for both the CELSS program and terrestrial agriculture.

Publications

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NASA GRADUATE STUDENT RESEARCHERS PROGRAM

In 1980, NASA initiated the Graduate Student Researchers Program (GSRP) in order to cultivate additional research ties to the academic community and to support promising students pursuing advanced degrees in science and engineering. Since then, approximately 800 students have completed the program's requirements while making significant contributions to the nation's aerospace efforts. Universities have also benefitted through strengthening their research capabilities.

Each year, NASA selects approximately 80 new students for the opportunity to receive stipends and to work at the unique national laboratories of the NASA facilities or at their home universities. Awardees are selected based on competitive evaluation of their academic qualifications, their proposed research plan, and their planned use of NASA research facilities. Fellowships are awarded for one year and are renewable, based on satisfactory progress, for up to three years.

The Graduate Student Researchers Program is managed at NASA Headquarters by the University Programs Branch, Educational Affairs Division. Forty of the 80 new awards each year are sponsored by the NASA Headquarters Office of Space Science and Applications (OSSA) in the fields of life sciences, astrophysics, earth sciences, solar system exploration, and microgravity science and applications. OSSA fellows carry out their research or a plan of study at their home universities. Each year they attend a two- or three-day annual symposium at NASA Headquarters in Washington, D.C. The symposium provides an opportunity for GSRP fellows to exchange ideas, discuss progress, and learn more about space science and applications at NASA.

The remaining 40 new awards are distributed throughout the NASA field centers. Fellows selected by centers must spend some time in residence at the center, taking advantage of the unique research facilities of the installation and working with center personnel.

Of the awards currently sponsored by the Headquarters Office of Space Science and Applications, thirteen are in the space biology and biomedical research areas. These awardees' abstracts, which were compiled from the May 1989 annual symposium in Washington, D.C., are included in the following pages.

Further information about the OSSA Graduate Student Researchers Program may be obtained from: Mr. Joseph K. Alexander, Assistant Associate Administrator for Space Science and Applications, Code E, NASA Headquarters, Washington, D.C. 20546. Mr. Gary Gans or Mr. John Lynch, University Programs, Educational Affairs Division, Code XEU, NASA Headquarters, Washington, D.C. 20546, may be contacted for further information about the 40 GSRP fellowships to conduct research at NASA facilities and centers.

ROLE OF VASOPRESSIN IN BAROREFLEX ALTERATIONS FOLLOWING SIMULATED WEIGHTLESSNESS

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Loss of hydrostatic pressure gradients upon exposure to weightlessness is associated with a shift of intravascular and interstitial volume to the thorax. The ensuing stimulation of cardiopulmonary mechanoreceptors results in a decrease of blood volume. Arginine vasopressin (AVP) is one hormone involved in volume regulation, and its release is diminished during weightlessness. However, upon return to gravity, redistribution of blood volume and the state of volume contraction likely enhance AVP release. In addition to affecting blood volume, AVP modulates blood pressure control via effects on the baroreceptor reflex (BRR). The goal of this study was to determine whether changes in AVP release associated with establishment and release from simulated weightlessness affect sensitivity of the BRR. Experiments were performed on conscious, chronically instrumented rats in which weightlessness was simulated by tail suspension using modified methods of Morey (*Bioscience* 29: 168-172, 1979). In an initial group of rats, plasma volume was monitored following initiation of simulated weightlessness to determine the time required for values to stabilize and therefore the appropriate time to assess BRR sensitivity. Rats of a parallel group were instrumented for determination of hemodynamic changes associated with simulated weightlessness. Immediately prior to hindlimb unloading, control cardiac output (CO), mean arterial blood pressure (MABP) and heart rate (HR) were determined. In addition, BRR sensitivity was assessed both before and after blockage of vasopressinergic receptors to determine the contribution of AVP under control conditions. Throughout the period of hindlimb unloading, MABP, HR, and CO were monitored daily. BRR sensitivity was assessed with and without vasopressinergic blockade on the last day of unloading to determine the role of AVP in BRR function during simulated weightlessness. Finally, following release from hindlimb unloading, the hemodynamic variables were monitored, and BRR sensitivity again assessed before and after vasopressinergic blockade. The data presented will demonstrate alterations of hemodynamics and BRR associated with adaptation to and release from simulated weightlessness as well as the contribution of AVP to these changes.

GRAVITROPISM MUTANTS IN THE CRUCIFEROUS PLANT, *ARABIDOPSIS THALIANA*

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Little is known about the sequence of events which result in gravitropism in plants. We have chosen to dissect the gravitropism pathway using mutants of the small crucifer, *Arabidopsis thaliana,* and an experimental system which allows us to examine root and hypocotyl curvatures simultaneously. Because of the plasticity of the wild-type response, we have subjected 60 putative mutants to a tertiary screen. Four mutants have been selected from this tertiary screen for further physiological and genetic analyses. Three of these mutants fail to respond to the gravity vector and are probably receptor or early transduction mutants. The fourth mutant exhibits an altered gravitropic response and may prove useful in examining the identities and interactions of the plant growth regulators involved in gravitropism.

AUXIN PHYSIOLOGY OF THE TOMATO MUTANT *DIAGEOTROPICA*

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The tomato (*Lycopersicon esculentum,* Mill.) mutant *diageotropica* (*dgt*), exhibits biochemical, physiological, and morphological abnormalities which suggest these plants may have a defect associated with a primary site of auxin perception or action. We have investigated aspects of the auxin physiology and growth parameters of *dgt* and wild-type (VFN8) seedlings. The rate of basipetal indole-3-acetic acid (IAA) polar transport is identical in the two varieties, but *dgt* sections have a slightly greater capacity for IAA transport. 2,3,5-triiodobenzoic acid (TIBA) and ethylene reduce transport in mutant and wild-type sections. The kinetics of auxin uptake into VFN8 and *dgt* sections are nearly identical. These results make it unlikely that an altered IAA efflux carrier or IAA uptake symport are responsible for the pleiotropic effects resulting from the *dgt* mutation. Measurements reveal that the osmotic potential of *dgt* cells is more negative than fluid expressed from VFN8. Thus turgor in *dgt* cells is adequate to drive cell extension suggesting this growth parameter is not responsible for the inability of *dgt* sections to elongate in response to IAA. Auxin treatment causes an increase in plastic wall extensibility (i.e., wall loosening) in VFN8 sections but has no effect on *dgt* sections. These data imply *dgt* hypocotyls suffer a defect which prevents them from initiating a key event which culminates in auxin-induced cell wall loosening.
(Additional authors: D.L. Rayle, R.E. Cleland)

CHARACTERIZATION OF CALCIUM-STIMULATED PROTEIN KINASES FROM ZUCCHINI AND TOMATO HYPOCOTYL PLASMA MEMBRANES

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Phosphorylation is an important mechanism by which proteins are regulated in a cell. For example, signal transduction in animal cells is a process that requires precise regulation of protein activity and is mediated by the action of protein kinases. A similar type of phosphorylation-dephosphorylation mechanism has been suggested to play a role in plant gravitropism. Our laboratory has previously identified several calcium-dependent protein kinases from zucchini hypocotyls. We have now developed a nitrocellulose autophosphorylation assay in which membrane proteins are fractionated by SDS-PAGE and blotted to a nitrocellulose filter. The immobilized proteins are then renatured, incubated with g-^{32}P-ATP, and autoradiographed. Comparisons with a -^{32}P-ATP have been used to demonstrate that this is a covalent protein modification rather than a non-specific interaction between ATP and protein. With this technique we have begun to characterize the kinases from zucchini and tomato as to the effect of pH and gravistimulation on autophosphorylation activity. Activity is maximal at pH 7.8 and there appears to be a reduction in autophosphorylation after gravistimulation of etiolated hypocotyls. This response is being investigated further as to presentation time and tissue-specificity. Whether or not the kinases are related will be investigated with the use of nitrocellulose-phosphorylation of proteins which have been separated on two-dimensional gels. This will also allow determination of the isoelectric point, as well as a first step toward isolation, of one or more of these enzymes. Gravitropic mutants from tomato are also being investigated for protein autophosphorylation.

GRAVITATIONAL STRESS AND LIGNIFICATION IN AERIAL VS. SUBMERGED SHOOTS OF *HIPPURIS VULGARIS*

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Hippuris vulgaris is a heterophyllic aquatic plant that grows naturally under the different degrees of gravitational stress that are associated with underwater and above water environments. This characteristic of *H. vulgaris* was exploited in order to study the interaction of gravitational stress with lignification processes. Lignin content was found to be 4.1% of aerial stem dry weight and 2.6% of submerged stem dry weight. The activity of phenylalanine ammonia-lyase, an enzyme early in the lignin biosynthetic pathway, paralleled lignin content and was about 5 times higher in aerial than in submerged stems. Another lignin biosynthetic enzyme, peroxidase, was studied and although definite conclusions could not be drawn from measurements of total peroxidase activity, different isozyme patterns were observed in aerial- and submerged-type shoots. Abscisic acid, which can induce the aerial-type shoot morphology on submerged shoots, probably is not involved in mediating changes in the lignin content of *H. vulgaris*. These results support the hypothesis that lignin biosynthesis is regulated by gravity.

EXPRESSION OF CELLULAR STRESS PROTEINS IN REPRODUCTIVE TISSUES

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The long-term goal of this project is to assess the effects of space flight on mammalian germ cell development and early embryogenesis at the molecular level. As molecular markers of both normal cellular differentiation as well as cellular response to stress, we have chosen to examine the expression of cellular stress protein genes, also known as heat shock protein genes (hsp). The initial part of this study has focused on characterizing the expression of the hsp90 family in the mouse testis. We have used a cDNA probe homologous to a human stress inducible transcript and have observed an abundant 3.2kb transcript in the mouse and human testis. To determine the developmental specificity of expression of this hsp90 gene in mouse testis, RNA was isolated from mice at different developmental stages of postnatal development. The levels of the 3.2kb hsp90 transcript were lowest in day 7 testis, which contain only premeiotic germ cells; increased in day 17 testis, which contain germ cells in all stages of meiotic prophase; and were most abundant in the adult testis, which contains the complete germ cell lineage from spermatogonia to spermatozoa. This suggested that the increased levels of hsp90 mRNA correlated with the presence of differentiating germ cells. Analysis of enriched populations of germ cells demonstrated that this transcript is present in all stages of germ cells, from pachytene spermatocytes to residual bodies. RNA was isolated from testes of germ cell deficient mice of the (W/W^V) genotype and from the testes of their fertile wild type littermates ($+/W^V$). The presence of the 3.2kb transcript in the testis of the wild type and mutant mice suggest that the hsp90 transcript is present in both the germ and the somatic cells. These observations demonstrate that the hsp90 mRNA is under developmental regulation in the mouse testis and would be useful as a sensitive molecular indicator of cellular stress.

BONE FORMING CELLS AND THEIR PROGENITORS IN RESPONSE TO SIMULATED SPACEFLIGHT

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It has been demonstrated that the microgravity environment of spaceflight is associated with a decrease in normal bone mass in both humans (*Acta Astron.* 6: 1113-1122, 1979) and rodents (*Science* 201: 1138-1141, 1978; *Am. J. Physiol.* 244: R319-R326, 1983). Immobilization produces a similar effect. In rats, this decrease appears to result from a reduction in normal patterns of bone formation.

Bone formation requires the proliferation and differentiation of the osteoblast precursor cells (OPCs) (which are presumable components of the bone marrow stromal system) (*Calcif. Tiss. Int.* 36: S37-S45, 1984). Employing the hindlimb unloading rat model to simulate spaceflight (*Metab. Bone Dis. & Rel. Res.* 4: 69-75, 1982; *Endocrinol.* 114: 2264-2270, 1984), we studied the effect of skeletal unloading on the clonal growth of marrow OPCs (cultured for 10 days in -MEM supplemented with 10% fetal bovine serum) and the behavior of osteoblast resident to the bone marrow-endosteum interface (quantified by vital tetracycline labelling; 10 mg/Kg).

Following 3 to 18 days of simulated spaceflight, there was a consistent decline in stromal cell colony number after 8 days ($p<0.001$) persisting through 18 days ($p<0.001$). Skeletal unloading also results in a reduction in bone formation by 1 week ($p<0.001$), but normalizes between the second and third weeks. We conclude that this may indicate a lag between stromal cell proliferation and osteoblast function and/or the enhanced production (by the stromal cells) of some growth factor(s) active on endosteal osteoblasts.

Studies designed to investigate the response of OPCs to recovery from hypokinesia, as well as the effects of sodium fluoride administration, are currently underway.

FRACTIONAL ACTIVITIES OF GLYCOGEN SYNTHASE AND PHOSPHORYLASE DURING RECOVERY OF THE 72-HOUR UNLOADED SOLEUS

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To study the mechanism of the triphasic change in muscle glycogen with reloading, we measured fractional activities of glycogen phosphorylase (GP) and glycogen synthase (GS) since they may reflect more precisely *in vivo* glycogen metabolism. Activities were determined at various times of reloading up to 72 h using the concentrations of the allosteric effectors for GP (AMP) and for GS (glucose-6-phosphate; G6P) at each time. During acute recovery muscle glycogen decreased by 21% in 15 min, reaching a maximum decline of 44% by 2 h. Muscle G6P increased concurrently with this net glycogenolysis. The fractional activities of GS and GP increased at 15 min and remained elevated through 2 h. Net glycogenesis occurred between 2 and 12 h of recovery resulting in a 2-fold increase in glycogen relative to the minimal value at 2 h. Over this same period, muscle G6P fell. Associated with this net glycogenesis GS fractional activity rose further, while GP fractional activity declined at 4 h. The final phase of recovery (24-72 h) showed net glycogenolysis, with muscle glycogen returning to normal by 72 h. The GP fractional activity increased at 12 h and remained elevated through 48 h before falling to normal at 72 h. The GS fractional activity decreased slightly between 12 and 24 h and returned to normal by 72 h. These results show that changes in the fractional activities of GP and GS can explain the triphasic response of glycogen during recovery from unloading. (Additional authors: E.J. Henriksen, W.J. Hartshorne, S.M. McCready)

PHYSIOLOGICAL RESPONSE TO VISUALLY INDUCED MOTION SICKNESS

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The development of techniques for the measurement of motion sickness is part of the effort to identify the mechanisms involved in the space adaptation syndrome and to provide a basis for predicting which countermeasures are the most appropriate preparation for spaceflight. The current study tested several measures of physiological response to visually induced motion sickness. These measures included heart rate, blood pressure, respiration, stomach activity, and peripheral blood flow.

In some situations, researchers find a relationship between subjective symptoms of motion sickness and measures of heart rate and blood pressure (Cowings, Billingham, and Toscano, 1977), while in others they do not (Hemingway, 1945; Graybiel, et al., 1960; Graybiel and Lackner, 1977). A similar amount of variability has emerged for the respiratory response (McEachern, Morton, and Lehman, 1942; Money, 1970). Most of these measures were not strong indicators of motion sickness in the current study. Only systolic blood pressure showed a slight rise during the period of visual stimulation.

Stern, et al. (1985) observed a rise in the frequency of electrogastrogram recordings during visually induced motion sickness. The current study used a similar experimental protocol. Although comparable levels of motion sickness were elicited, an increase in frequency of electrogastrogram was not as readily confirmed.

Sunahara, Johnson, and Taylor (1964) and Sunahara, et al. (1987) observed an increase in peripheral blood flow during motion sickness induced by head movements during rotation. No large changes in blood flow to skeletal muscle were observed during the visual stimulation period in this study; however, there was some tendency for blood flow to the skin to change slightly over the course of the experiment.

EFFECT OF LOADED ISOTONIC CONTRACTIONS ON MUCLE MASS, STRENGTH, AND FATIGABILITY IN DISUSE ATROPHIED RAT SKELETAL MUSCLE

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Disuse atrophy of skeletal muscle results from whole body suspension (WBS) and is characterized by reductions in muscle mass and absolute muscle strength. In an attempt to prevent such losses, hind limb skeletal muscle from suspended rats was subjected to maximal isotonic contractions every 48 hours. Fourteen days of WBS, without muscle exercise, resulted in significant reductions in soleus (-41%), plantaris (-20%), and gastrocnemius (-14%) muscle masses. Isotonic exercise fully attenuated plantaris and gastrocnemius muscle atrophy, but failed to alter soleus atrophy (-31%). Absolute twitch and tetanic isometric tensions declined 29% and 22%, respectively, in WBS controls while relative tensions (g/g muscle mass) were unchanged. Isotonic exercise of WBS animals resulted in no change in absolute or relative twitch or tetanic strength. Gastrocnemius muscle fatigue was significantly ($p<0.05$) greater in weight matched cage controls than in WBS and WBS + isotonic exercise animals after 1 min of continuous contractile activity. Results of this study indicate that isotonic exercise training prevents atrophy and strength losses of highly fast twitch muscles experiencing disuse. However, atrophied skeletal muscle functions normally per unit mass, despite a loss in overall contractile tissue. Metabolic alterations within the fibers appear to have resulted in greater fatigue resistance in atrophied muscle while further fatigue resistance is developed in exercise-trained animals.

THE EFFECTS OF ANTIORTHOSTASIS (HEAD-DOWN TILT) ON BODY TEMPERATURE RHYTHMS

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This experiment examined the effect of antiorthostasis (head-down tilt) on the core body temperature rhythm in rats. Head-down tilt has been proposed as a ground-based model for the study of the effects of weightlessness. Head-down tilt is believed to simulate the headward fluid shifts seen in a microgravitational environment, where gravity no longer pulls blood into the extremities. Changes in core body temperature rhythm have been seen in rats during spaceflight and in hypergravitational environments. We hypothesize that head-down tilt will increase the length of the temperature cycle as is seen during spaceflight.

In this study we used hindlimb unloading to maintain the head-down tilt position in the rats. The body temperature was monitored using telemetry transmitters, which were implanted in the peritoneal cavity. A receiver below the cage relayed the temperature data to a microcomputer, which recorded data every ten minutes. The rats were visually isolated in an environmentally controlled room.

During both the control and the antiorthostatic periods, core body temperature showed the characteristic elevation during the dark hours, but there appeared to be changes in the time at which the peak temperature occurred. Further data analysis will be completed before the symposium.

EFFECTS OF FLUID LOADING ON HEMODYNAMICS, EXTRACELLULAR FLUID MOVEMENT, AND ATRIAL NATRIURETIC PEPTIDE

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We hypothesized that atrial natriuretic peptide (ANP) participates in the cardiovascular response to excess fluid volume by enhancing vasodilation and fluid shifts. To test this hypothesis, we measured cardiovascular, extracellular fluid, and ANP secretory responses of 8 subjects to infusion of 30 ml/Kg isotonic saline.

Sphygmomanometry provided blood pressure (MAP, mmHg). Acetylene rebreathing yielded cardiac output (Q, l/min). Right atrial pressure (RAP, mmHg) was measured via a central catheter. Systemic vascular resistance (SVR, mmHg x min/l) equals (MAP - RAP) / Q. Venous occlusion provided leg capillary pressure (CP, mmHg). Plasma volume (PV, ml) was measured with Evans blue dye, and later calculated from hematocrit. Changes in plasma volume not attributable to urine formation equalled net whole body filtration rate (NFR, ml/h). Urine production was measured volumetrically or with ultrasonic bladder imaging. Plasma ANP (pmol/l) was measured by radioimmunoassay. Observations were made prior to infusion, at the end of infusion, and at 30, 60, 120, and 180 min post-infusion. Data were analyzed with repeated measures ANOVA (alpha = 0.05) and paired T-tests of differences from control (*: alpha = 0.01). Data are expressed as mean/standard error.

Saline infusion acutely decreased SVR, which demonstrates a systemic vasodilation. Infusion produced a profound filtration of fluid from the vasculature by elevating CP and diluting plasma proteins. Within 1 h, however, NFR became negative in all but 1 subject, thereby reflecting a reabsorption of fluid from extravascular spaces. Observed increases in ANP were not significant.

	Control	End Inf.	+30 min	+60 min	+120 min	+180 min
RAP	5.3/0.8	12.4/1.0*	7.5/0.9*	7.6/0.9*	7.1/1.0	6.3/0.8
CP	24/1	28/1*	24/1	23/1	24/1	23/2
SVR	13.6/0.8	9.7/0.5*	10.9/0.6*	12.0/0.5	12.6/0.6	12.9/0.5
PV	3200/196	4012/274*	3549/213*	3505/186*	3491/188*	3390/168
NFR	-106/24	1229/124*	469/174	-236/102	-222/48	-114/54
ANP	29/1	35/4	42/3	37/3	35/3	34/2

The early transudation of fluid accompanied by vasodilation attenuated volume-induced cardiovascular pressure elevations, and subsequent reabsorption of fluid allowed slow restoration of baseline conditions while the kidneys disposed of the excess fluid. Since changes in ANP were insignificant and not associated with vasodilation or filtration, ANP is probably not a prominent mediator of the cardiovascular response to excess intravascular fluid.

THE DIFFERENTIATION OF TRACHEARY ELEMENTS IN LEAF DISCS OF *ZINNIA ELEGANS* SUBJECTED TO CLINOSTATING

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Life on earth has evolved under the influence of gravity. As a result, the degree to which developmental processes depend on gravity cannot be readily assessed in a gravity environment. In an attempt to distinguish the effect of gravity on cellular differentiation in plants, the sensitivity of differentiating parenchyma cells to a simulated microgravity environment was investigated. Tissue discs derived from young leaves of the Composite, *Zinnia elegans,* can be induced to differentiate as tracheary elements on defined media containing the plant growth regulators, auxin and cytokinin. Cells differentiating as tracheary elements undergo a process of secondary wall formation, lignification, and autolysis homologous to that of the conducting cells of the xylem found in all vascular plants. When *Zinnia* leaf discs are subjected to clinostating, tracheary element differentiation proceeds in a similar manner to that observed in control discs. Secondary wall thickenings can be detected within 8 hours after the start of culture leading to the formation of mature tracheary elements by 96 hours. While the absolute number of tracheary elements formed in control discs represents approximately 20% of the total retrievable cell population, a particular subpopulation of cells, the paraveinal parenchyma, displays enhanced recruitment into the differentiation pathway in clinostated leaf discs. It is hypothesized that clinostating imposes a stress on the leaf disc which indirectly enhances the extent of paraveinal parenchyma differentiation.